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Applicants	DePhillipo and Ricciardi
Serial No.	09/924,011
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Confirmation No.	1968
Examiner	Kaushal
Title	Use Of Genetic Information For Detecting a Bone Density Condition
Attorney Docket No.	073981.2

Cincinnati OH November 16, 2007

DECLARATION OF ROBERT P. RICCIARDI, Ph.D.
PURSUANT TO 37 C.F.R. §1.132

I, Robert P. Ricciardi declare as follows:

1. I am an inventor in the above-identified patent application.

2. I received my Ph.D. degree in Cellular and Molecular Biology from the University of Illinois in 1977. I have over 30 years of experience in this field, which is the subject of this application. I have read the outstanding Office Action and understand the Examiner's position.

3. I understand that the Examiner rejects my claims as indefinite, and rejects my application as lacking sufficient description and enablement. My Declaration addresses each individual basis of the Examiner's rejections.

4. My claimed method, as currently amended, reads as follows; I have underlined the terms the Examiner deemed unclear:

> A method comprising
>
> assessing a relative degree to which a human is susceptible to an undesirable bone density condition by identifying a polymorphic form identified as associated with a bone density pathology in each of
>
> a gene encoding a vitamin D receptor present in the human's genome, and
>
> a gene encoding interleukin-6 present in the human's genome,
>
> thereafter calculating a susceptibility value for the condition by either

summing the identified polymorphisms to yield a value for the human, or

assigning a weighting factor to each polymorphism and then summing the weighting factors to yield a value for the human,

wherein a value for the human greater than a value for a control indicates a greater susceptibility to the undesirable bone density condition for the human, and wherein the polymorphic form identified as associated with any bone density pathology is a disorder associated polymorphism,

the method hereby assessing the relative degree to which the human is susceptible to the undesirable bone density condition.

The Examiner states that "it is unclear what encompasses calculating a susceptibility value for the condition by either summing the identified polymorphisms to yield a value for the human, or assigning a weighting factor to each polymorphism and then summing the weighting factors to yield a value for the human". I disagree, because using available information or information obtained without undue experimentation, one of ordinary skill in the art can calculate a susceptibility value for these polymorphisms. For any polymorphisms that do not have a known value, my method assigns a value of 1.0. I now describe each of these steps in further detail.

5. I disagree that summing the identified polymorphisms to yield a value for the human is unclear.

6. As I disclose in my application, the mere presence of a polymorphism in a gene encoding a Vitamin D receptor and a gene encoding interleukin-6 (IL-6) has predictive value to one of ordinary skill in the art. Even if no additional information is known about it, such as exactly how it correlates to a bone density condition, as long as it is present, it is assigned a value of 1.0. These assignments occur for each polymorphism in each of the Vitamin D receptor and IL-6 genes. Also, as an example of my method, see Appendix A which provides data and analysis of an individuals' susceptibility to oxidative stress.

7. The values are then summed (i.e., added). The summed value is a value for the human.

8. I disagree that assigning a weighting factor for each polymorphism and then summing the weighting factors to yield a value for the human is unclear.

9. Some polymorphisms are known to one of ordinary skill in the art as highly associated with a bone density condition. The presence of such polymorphisms, therefore, has greater predictive value. To account for the increased predictive value of these highly associated polymorphisms,

my method assigns a weighting factor. The weighting factors for each polymorphism present in each of the Vitamin D gene and the IL-6 gene are then summed to yield a susceptibility value.

10. My application discloses how to assign a weighting factor to each such highly predictive polymorphism.

weighting factor = (correlation factor) (constant)

11. The "correlation factor" is determined by how closely the polymorphism correlates with the bone density condition, as known to one of ordinary skill in the art. For example, a human that is homozygous for the polymorphism will have a higher correlation factor than a human that is heterozygous for the polymorphism (e.g. ¶16).

12. The "constant" is determined by how relevant the polymorphism is to the bone density condition, as known to one of ordinary skill in the art. As I discloses at ¶16, one way to determine the "constant" is by an "odds ratio" (also known as "relative risk").

odds ratio = odds of a bone density condition occurring in one group : odds of the same bone density condition occurring in another group

The groups compared are humans who (a) lack the polymorphic allele, (b) are heterozygous for the polymorphic allele, or (c) are homozygous for the polymorphic allele. Thus, the "correlation factor" and "constant" are derived from data known by or available to one of ordinary skill in the art.

13. The weighting factor is then assigned. As one example, Gennari et al., which I cite, demonstrates how my method may be used. Gennari correlates a polymorphism in the vitamin D receptor gene with osteoporosis, a known bone density condition. Specifically, he finds a statistically significant two-fold increase in the prevalence of a T/C polymorphism in the initiation codon of the human vitamin D receptor gene in patients with osteoporosis, compared to patients without osteoporosis (p. 1382). Information such as this also provides an example of how to calculate my claimed "correlation factor". For example, see Gennari Table 4 where

number of homozygous patients exhibiting a bone density disorder = 44
total number of homozygous patients = 55
"correlations factor" is 44/55 = 0.80

My claimed "constant" is calculated using the relevance between the polymorphic allele and a bone density condition, as shown in Table 5. For example, using the presence of vertebral fractures in patients, which indicates an undesirable bone density condition, 25% of patients having vertebral fractures were homozygous for the bone density disorder-associated polymorphism. Conversely, only 11% of patients not having vertebral fractures were homozygous for the bone density disorder-associated polymorphism.

14. Using this information, Gennari reports an odds ratio of 2.58 for homozygous patients and 1.59 for heterozygous patients. A person of ordinary skill in the art calculates or obtains the "correlation factor" and the "constant", then calculates the weighting factor as follows:

weighting factor of homozygous polymorphism = (correlation factor) (constant)
= (0.80) (2.58)
= 2.1

In my method, the weighting factor is determined for each polymorphism in each of the vitamin D receptor gene and the IL-6 gene in the individual.

15. All the weighting factors are summed to "yield a value for the human" which "assess his/her relative degree to which he/she "is susceptible to an undesirable bone density condition", as I claim.

16. I disagree with the Examiner that the metes and bounds of the control are unclear in my claims that a value for the human greater than a value for a control indicates a greater susceptibility to the undesirable bone density condition for the human.

17. A value for a control can be derived from either an individual other than the test individual, or in a hypothetical individual. For example, the susceptibility value in a hypothetical individual can be assumed to be 0. Thus, for each polymorphism in each of a gene encoding a vitamin D receptor and a gene encoding IL-6 in the test individual, the individual's susceptibility to a bone density condition increases relative to the hypothetical individual. In this way, even if the correlation factor and constant are not known for a particular polymorphism, the presence of the polymorphism alone can access susceptibility to a bone density condition in each of the vitamin D receptor gene and the IL-6 gene.

18. I disagree that the oligonucleotides encompassing a disorder associated polymorphism and non-disorder associated polymorphism are unclear.

19. My claimed method requires

> assessing the occurrence of an individual disorder associated polymorphism by contacting a nucleic acid derived from the human's genome with a first oligonucleotide that anneals with higher stringency with the disorder associated polymorphism than with a corresponding non-disorder associated polymorphism, and
>
> assessing annealing of the first oligonucleotide and the nucleic acid,

whereby annealing of the first oligonucleotide and the nucleic acid indicates that the human's genome comprises the disorder associated polymorphism.

20. One of ordinary skill in the art knows that oligonucleotide annealing is a specific assay. A first oligonucleotide specific for a bone density associated polymorphism in each of a vitamin D receptor gene and an IL-6 gene is known to anneal with higher stringency to these polymorphisms that to other sequences. Therefore, one of ordinary skill in the art would know the metes and bounds of a first oligonucleotide that anneals with higher stringency with the disorder associated polymorphism than with a corresponding non-disorder associated polymorphism, as my claim requires.

21. Assessing the relative degree to which a human is susceptible to an undesirable bone density condition, as I claim, requires determining whether a polymorphism in each of the vitamin D receptor and the IL-6 gene is present. Their presence is assessed by contacting a nucleic acid derived from the human's genome with a first oligonucleotide that anneals with higher stringency with the disorder associated polymorphism than with a corresponding non-disorder associated polymorphism, as my claim requires. A second oligonucleotide that anneals with higher stringency with a non-disorder associated polymorphism than with the corresponding disorder associated polymorphism may also be used.

22. For an example of oligonucleotides encompassing disorder associated polymorphism and non-disorder associated polymorphisms, continuing to use Gennari's example, a first oligonucleotide would preferentially bind to the polymorphic sequence containing T, and a second oligonucleotide would preferentially bind to the polymorphic sequence containing C. Using this method, the presence of the polymorphism is detected, as well as whether the polymorphism is homozygous or heterozygous, i.e., whether 0, 1, or 2 of the alleles contains the disorder associated polymorphism. Determining homozygosity of a bone density associated polymorphism "assesses the relative degree to which the human is susceptible to the undesirable bone density condition", as I claim.

23. I disagree that my application "fails to disclose first and second oligonucleotides especially in context of any and all variants gene encoding vitamin D receptor and interleukin-6". One of ordinary skill in the art recognizes that, using known polymorphisms identified as associated with a bone density pathology in each of the vitamin D receptor gene and IL-6 gene, he/she can create substantially complimentary oligonucleotides to the polymorphic forms of each of these genes, as I subsequently explain.

24. One of ordinary skill in the art knows or can determine sequences of gene variants of each of the vitamin D receptor gene and IL-6 gene. For example, publicly available searchable databases such as Genbank, SwissProt, and GenCards provide this information, as well as polymorphisms in each of the vitamin D receptor gene and IL-6 genes. Publicly available searchable databases such as HapMap Project, dbSNP, SNP500 Cancer, GeneSNPs, Seattle SNPs, regulatory-rSNP, and SNPtagger also provide polymorphisms. As only one example, the vitamin D receptor page on GenCards (http://www.genecards.org/cgi-bin/carddisp.pl?gene=VDR&search=vitamin+D+receptor&suff=txt) displays known single nucleotide polymorphisms (SNPs). Association of these SNPs with bone density associated disorders can also be found in databases such as OMIM (Online Mendelian Inheritance in Man), AKS (almaKnowledgeServer), and PharmGKB. These sites show vitamin D receptor SNPs associated with osteoporosis and bone resorption, along with the sequences and related scientific reference. Thus one of ordinary skill in the art can readily determine, without undue experimentation, the sequences of each of the vitamin D receptor gene and the IL-6 gene, as well the sequences of the polymorphisms associated with each of these genes. Thus, one of ordinary skill in the art can use these data to generate a first and second oligonucleotide, as my claim requires.

25. I disagree with the Examiner that "demonstration of an association between a candidate gene [polymorphism] and [a bone density related disorder] does not necessarily mean that the gene [polymorphism] is causally responsible for the effect observed".

26. I claim that the susceptibility value allows assessing the relative degree to which the human is susceptible to the undesirable bone density condition. One of ordinary skill in the art recognizes that the problems with "confounding factors" and "population stratification" are largely negated by techniques known in the art. For example, Ferrari et al., which I cite in my application, addresses potential problems in the association between IL-6 polymorphisms and bone density associated disorders by evaluating data using Kruskal-Wallis analysis, analysis of variance (ANOVA), and standard linear regressions. Confounding factors (e.g., dietary and clinical variables) are also evaluated using, e.g., logistic regression at a 95% confidence interval. Thus, the association between the polymorphism and the bone density condition disorder can be established.

27. I disagree that my application "fails to provide any evidence, which establishes that assessment of these SNPs in combination would be a better predictor of assessing an undesirable bone density [disorder] as compared to identification of a single SNP". My claims

require assessing a human's susceptibility to an undesirable bone density condition by identifying a polymorphic form in each of a vitamin D receptor gene and an IL-6 gene.

28. One of ordinary skill in the art recognizes that undesirable bone density conditions (using osteoporosis as only one example) are multi-genetic pathologies (e.g., undesirable bone density conditions implicate other genes, such as type I collagen gene and estrogen receptor gene as well as the vitamin D receptor gene and the IL-6 gene). Therefore, assessing the presence of multiple SNPs better assess the susceptibility of an individual to undesirable bone density conditions.

29. I understand the Examiner questioned how my method controls for variation found in various sub-populations, e.g., ethnic groups.

30. My method does not need to account for polymorphism frequencies in various sub-populations. In some cases, certain populations, e.g. ethnic populations, exhibit polymorphic allelic frequencies that are different from a general population. However, the presence of one of my claimed genetic polymorphisms indicates an increased susceptibility to an undesirable bone density condition. This increased susceptibility occurs whether the polymorphism is rare or common in a particular group, because my method assesses the relative susceptibility of a human to an undesirable bone density condition, not the frequency or probability that that human will develop a bone density condition. For example, ethnic group A has a very low frequency of bone density associated polymorphism P. If a human which belongs to ethnic group A exhibits polymorphism P, then my method indicates that this human is relatively more susceptible to a bone density pathology. The frequency of the polymorphism in a population is only important in determining the absolute probability that that individual will develop a bone density related disorder.

31. I understand the Examiner questioned how my method would account for SNPs that increase, versus decrease, activity or expression of the encoded protein.

32. SNPs that increase activity or expression of the encoded protein are very rare. My method accounts for the rare occurrence of polymorphisms that result in an increase in activity or expression of the encoded protein. As amended claim 65 recites, the method is accomplished by "... identifying a polymorphic form identified as associated with a bone density pathology ...". Thus, it is the presence of the polymorphic form associated with a bone density pathology that is used to assess the individuals susceptibility. In the case where a polymorphism increases activity or expression of the encoded protein, then the polymorphic form is not associated with the bone

density pathology. In this case, the non-polymorphic form of the gene would be the form that is associated with a bone density pathology and therefore used in assessing susceptibility. For example, a polymorphism that is defined by allele "A" and allele "a" exhibits two different expression levels or activities for the protein encoded by the gene containing polymorphisms "A" and "a". Whether "A" has greater activity or expression than "a", or vice versa, is inconsequential. My method requires the identification of the polymorphic form that is associated with a bone density pathology, and then uses the presence of that polymorphism to assess susceptibility.

33. For at least the above reasons, I believe my Declaration demonstrates that the claims are sufficiently clear and that my application sufficiently describes and enables my claimed method.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both , under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the subject application or any patent issued thereon.

November 15, 2007
Date

Robert P. Ricciardi
Robert P. Ricciardi, Ph.D.

664137

Appendix A – Proof of Concept

"Skin-Gene Profile Test of 177 Women"

Summary – A Gene-SNP skin profile of 177 women demonstrated that this test will be useful. The test highlights the high frequency of Gene-SNP markers for skin wrinkling, detoxification, and oxidative damage. By providing different levels of support, this Gene-SNP profile enables us to offer personalized skin care formulations that will act to help slow down or help prevent various types of damage to the skin.

The Process – Genelink collected DNA samples, via buccal swabs, from 177 individuals. The samples were genotyped and screened for SNPs in genes associated with Oxidative Stress. The frequencies of the SNPs in the test population are shown in Table 1. The Gene-SNP test data is used to create a profile describing an individuals relative susceptibility to various skin health problems and allows customization or personalization of products for each individual. Specifically, the Gene-SNP test recommends skin health areas where test individuals need additional support based on their particular susceptibility value.

Table 1. Gene SNP Frequencies (%)

Number of participants = 177

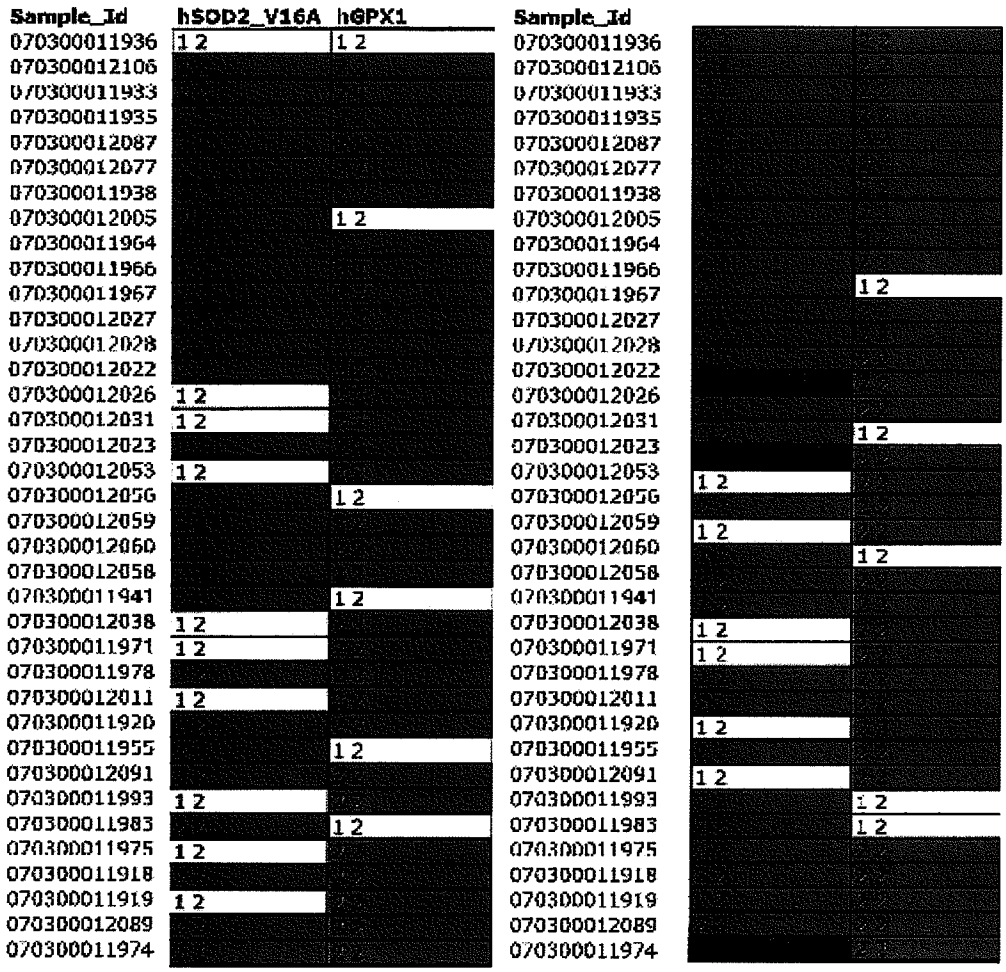
		Recommended Skin-Care Support		
Skin SNPs	**Functional Area**	**Maximum**	**Added**	**Basic**
		Red	Yellow	Green
MSOD (OS1)	Oxidative Stress	2%	29%	69%
GPX1 (OS2)	Oxidative Stress	1%	12%	88%

Interpreting Test Results

A GREEN test result (Homozygous Negative) predicts that you do not have any variant SNPs and that the protein molecules expressed by this given gene are functioning optimally to produce its specific enzyme, hormone, cytokine, or structural protein. A comprehensive BASIC SUPPORT skin-care formulation is recommended to keep this system functioning optimally.

A YELLOW test result (Heterozygous Positive) indicates that you have one variant SNP and that a portion of the protein molecules expressed by this given gene, are functioning suboptimally to produce a specific enzyme, hormone, cytokine or structural protein. In order to compensate for the less than optimal function of the protein molecule, ADDED skin-care support for this area is recommended.

A RED test result (Heterozygous Positive) indicates that you have two variant SNPs and that the protein molecule expressing a specific enzyme, hormone, cytokine or structural protein is functioning minimally. As a result, it is important to have a MAXIMUM SUPPORT skincare formulation in place to compensate for this inferior protein molecule.



A "1" indicates the presence of a disorder associated polymorphism and a "2" indicates the presence of a non-disorder associated polymorphism. The two numbers represent each allele of the gene. The hSOD2_V16A and hGPX1 genes are both associated with oxidative stress. The data shows for example, individual 11936 is more susceptible to oxidative damage than, for instance, individuals 12005 and 11964 which exhibit one and zero, respectively, of disorder associated polymorphisms in at least two genes associated with oxidative damage.

Conclusion – the Gene-SNP test is an excellent predictor for learning in advance the functional areas of skin health that need 'customized' care as a means to prevent future skin damage and wrinkling. By providing different recommended levels of support, this Gene-SNP profile enables personalized skin-care formulations that will act to help slow-down
or help prevent various types of damage to the skin.

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Vitamin D and Estrogen Receptor Allelic Variants in Italian Postmenopausal Women: Evidence of Multiple Gene Contribution to Bone Mineral Density

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ABSTRACT

Bone mass and bone turnover are under genetic control. Restriction fragment length polymorphisms (RFLPs) at the vitamin D receptor (VDR) gene locus have been recently correlated to bone mineral density (BMD) and rate of bone loss. However, agreement on this relationship is not universal. The existence of ethnical and environmental differences between populations, a health-based selection bias in several previous studies, and the involvement of other genes could explain these discordant findings. In this study, we examined the relationship of VDR and estrogen receptor (ER) gene RFLPs with lumbar spine and upper femur BMD in 426 Italian postmenopausal women, 57.7 ± 0.4 yr old (144 normal, 106 osteopenic, and 176 osteoporotic). VDR gene RFLPs for *Apa*I, *Bsm* I, and *Taq*I restriction endonucleases and ER RFLPs for *Pvu*II and *Xba*I restriction endonucleases were assessed by Southern blotting analysis and were indicated, respectively, as A-a, B-b, T-t, P-p, and X-x (uppercase letters signifying the absence and lowercase letters the presence of the restriction site). After correcting for potential confounding factors (age, height, weight, age since menopause, osteophytosis, and facet joint osteoarthritis), a statistically significant VDR genotype effect on lumbar BMD ($P = 0.01$, analysis of covariance), but not on femoral BMD, was detected, with subjects in AABBtt genotype showing a 13% lower BMD than those with aabbTT genotype ($P < 0.05$, Tukey's test). Moreover, a statistically significant prevalence of AABBtt genotype in osteoporotics, and of AabbTT and aabbTT genotypes in nonosteoporotics, were detected. Conversely, there was no significant relationship of ER genotype to either lumbar or femoral BMD, even though a trend for higher BMD values in women with the ER PP genotype (with respect to those with ER pp genotype) was detected. When mean lumbar BMD was calculated for women grouped by ER and VDR genotype, we observed a significant difference between those within the 2 opposite associations AABBtt-PPXX and aabbTT-ppxx (0.71 ± 0.05 *vs.* 0.97 ± 0.03 g/cm^2, $P < 0.05$ Tukey's test). These results are consistent with a segregation of the VDR AABBtt genotype with a higher risk of developing osteoporosis, in the Italian female population. The introduction of another variable, the ER genotype, in the analysis of VDR genetic determination of BMD, may represent a useful model in the identification of patients at risk of developing a multigenic disorder like osteoporosis. (*J Clin Endocrinol Metab* **83:** 939–944, 1998)

BONE mineral density (BMD), the major determinant of osteoporotic fracture risk, is a quantitative trait determined by the interaction of genetic, metabolic, and environmental factors. In the last years, both twin and family studies have suggested a major genetic contribution in bone mass determination and in the development of osteoporosis (1–4). The number of candidate genes is large, ranging from those regulating calcium homeostasis to the several locally involved in bone cell recruitment and activity. The vitamin D receptor (VDR) gene has been shown to be a major locus for genetic influences on bone mass, and polymorphisms in this gene seemed to predict spinal and femoral BMD in an Australian population (5). The subjects homozygous for the presence of the Bsm I restriction endonuclease site were reported to have a bone mass about 15% higher than that of subjects homozygous for the absence of the site (5). Successively, haplotype analysis, based on three restriction fragment length polymorphisms (RFLPs) at the VDR gene locus, showed the possibility to discriminate individuals at high and very low osteoporotic risk, with subjects homozygous for the abT haplotype showing lumbar BMD values 0.15 g/cm^2 greater than subjects homozygous for the ABt haplotype (6). However, agreement on this relationship is not universal among different populations, some finding positive associations (7–12), others reporting no significant effect (13–17).

The cause of the discrepancies remains to be determined, and in part, it may be caused by the limited sample size of many studies. Indeed, relationships between VDR gene alleles and osteoporotic risk, mediated through differences in BMD, are unlikely to be observed in relatively small samples. A potential confounder in all such studies may be given also from the health-based selection bias, with the tendency to exclude osteoporotic women. Currently, the few association studies, in which the prevalence of VDR genotypes in osteoporotic and nonosteoporotic patients are compared, have been carried out in small samples, with limited statistical power (12, 15, 16, 18). Heterogeneity also is likely, with different major genes segregating in different patient sam-

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ples. In this regard, other polymorphic genes, such as those encoding for estrogen receptor (ER), collagen type I α1, and interleuchin 6 recently have been linked to variation in BMD (19–21). These genes could either positively or negatively modulate VDR gene effect, with a different power, which needs to be individually evaluated. In addition, environmental factors also could reciprocally interact with genetic factors. Recently, dietary calcium intake has been reported to contribute to the expression of the VDR gene effect, both on BMD (10, 11) and on intestinal calcium absorption (22). Finally, linkage disequilibrium with another bone metabolism-related gene on chromosome 12 (*i.e.* Collagen 2 A1 and retinoic acid receptor genes) cannot be excluded.

Being aware of the constant interaction between genetic and environmental factors in bone mass determination, the present study was performed to evaluate, for the first time, in a large and ethnically homogeneous group of postmenopausal women of Italian descent (stratified for BMD in normal, osteopenic, and osteoporotic patients): 1) the relationship of VDR and ER gene polymorphisms with BMD, after controlling for multiple confounders; and 2) the possibility of an interaction between VDR and ER genotypes with bone mass, assuming that variation of BMD is influenced by multiple genes.

Subjects and Methods

Subjects

Patients eligible for the study were selected among 1500 consecutive postmenopausal women who, in 1995, attended the metabolic bone diseases outpatient clinics in Siena and Florence for osteoporotic risk evaluation. For all subjects, a detailed medical history was obtained, and dietary calcium intake was assessed by a sequential self-questionnaire, including foods that account for the majority of calcium in the diet. Among this group of women, 866 had associated conditions known to affect bone metabolism and were excluded from analysis. These were diseases known to influence bone mass (98 women), use of bone active drugs (303 estrogen replacement therapy, 198 vitamin D metabolite, 96 bisphosphonate, 59 calcitonin, and 7 fluoride) or use of drugs that could potentially affect bone metabolism (55 glucocorticoid, 34 thyroid hormone, and 16 antacid). Eighty-nine women also were excluded because of their different ethnical origin. Blood was available for DNA isolation in 426 of the remaining 545 subjects. The age range of the studied women was 47–76 yr, with a mean (±SEM) age of 57.7 ± 0.43 yr. On the basis of BMD measurements and according to WHO criteria (23), 40% of the 426 subjects had osteoporosis, 26% had osteopenia, and 34% were normal. General characteristics of the population are presented in Table 1.

Genotyping

Genomic DNA was isolated from EDTA blood samples by a standard phenol-chloroform extraction procedure, and 8 μg of DNA were digested for 6 h in a vol of 50 μL with, respectively, 40 U of *ApaI*, Bsm I, *TaqI*, *PvuII*, and *XbaI* restriction endonucleases (Boehringer Mannheim, Milan, Italy) at temperatures recommended by the manufacturer. The digested DNA was size-fractionated using 0.7–1% agarose gel electrophoresis and transferred to nylon-based filters (GeneScreen Plus, NEN Research Products, Boston, MA) by standard techniques (24). Filter membranes were prehybridized, then hybridized with the ^{32}P-labeled probe for 18 h at 65 C, washed, and autoradiographed for 24–48 h at −80 C in intensifying screen. For identifying the RFLPs, we used, as probes, a 2.1-kilobase complementary DNA (cDNA) coding region of the human VDR (25) and a 1.8-kilobase cDNA coding region of the human ER (26). The probes were radiolabeled with [α^{32}P]deoxycycidine triphosphate using a random priming labeling kit (Boehringer Mannheim, Milan, Italy). The RFLPs were coded as B-b (*Bsm* I), A-a (*ApaI*), T-t (*TaqI*), P-p (*PvuII*), and X-x (*XbaI*), uppercase letters signifying the absence and lowercase letters the presence of the restriction site.

Bone densitometry

Lumbar BMD (L2-L4), measured by dual-energy x-ray absorptiometry (Hologic QDR 1000/W) was available for all the 426 studied women. The long term *in vitro* precision at this site measured on spinal phantom was 0.4% in Siena and 0.6% in Florence; the *in vivo* precision was 0.9% in both centers. Dual-energy x-ray absorptiometry BMD scans at the upper femur were available for 230 of the 426 women (113 osteoporotic, 58 osteopenic, and 59 normal), with *in vivo* coefficients of variations of 1.1% in Florence and 0.9% in Siena. Cross-calibration studies on the precision of measurements between the 2 centers were previously performed both *in vitro* (using a single anthropomorphic lumbar spine phantom) and *in vivo*, on 50 patients, covering most of the clinically observed spinal density range. A correction factor was not considered necessary.

Because of the influence of extravertebral calcification on spinal bone mass measures, each woman underwent a lateral lumbar spine x-ray examination to be scored for spinal osteophytosis (SPO), according to Orwoll (27), and for facet joint osteoarthritis (FOA) on a four-point scale (0 = none, 1 = mild, 2 = moderate, and 3 = severe), according to Masud (28). Vascular calcifications were not evaluated for their reported limited impact on spinal density measurements (27–29).

Statistical analysis

Data were expressed as mean ± SEM, with $P < 0.05$ accepted as the value of significance. The frequency distribution of VDR genotypes in osteoporotic, osteopenic, and normal groups were compared using cross-tabulation and standard χ^2 tests. Differences in antropometric characteristic, spinal BMD, and femoral BMD among the different VDR and ER genotypes were tested using ANOVA. Similar comparisons were done after adjusting mean BMD values for potential confounding factors such as age, height, weight, years since menopause (YSM), SPO, and

TABLE 1. General characteristics of the study group patients

	Normal	Osteopenic	Osteoporotic	Total
n	144	106	176	426
Age (yr)	57.1 ± 0.7	57.9 ± 0.8	58.2 ± 0.6	57.7 ± 0.4
YSM	8.5 ± 0.9	9.0 ± 0.9	10.1 ± 0.7	9.3 ± 0.5
Height (cm)	163.9 ± 0.8[a]	161.6 ± 0.7	159.4 ± 0.6	161.4 ± 0.4
Weight (kg)	64.1 ± 1.3	64.5 ± 1.2	62.9 ± 1.2	63.7 ± 0.6
Ca intake (mg/day)	542 ± 142	568 ± 164	587 ± 128	567 ± 102
Lumbar BMD (g/cm^2)	1.05 ± 0.01[a]	0.88 ± 0.01[a]	0.69 ± 0.01	0.86 ± 0.01
SPO score	0.79 ± 0.07	0.73 ± 0.09	0.63 ± 0.10	0.70 ± 0.05
FOA score	0.63 ± 0.06	0.57 ± 0.08	0.57 ± 0.09	0.59 ± 0.04
Corrected BMD (g/cm^2)[b]	1.03 ± 0.01[a]	0.85 ± 0.01[a]	0.67 ± 0.01	0.83 ± 0.01

Values are expressed as mean ± SEM.
[a] $P < 0.05$ ANOVA *vs.* osteoporotic group.
[b] Lumbar spine BMD values, corrected for age, height, weight, YSM, SPO, and FOA scores.

FOA scores, using analysis of covariance. Between groups, differences among genotype groups were tested using Tukey's test. All statistical analyses were performed using STATGRAPHICS (Manugistic Inc., Rockville, Maryland) and Statistica 5.1 (Statsoft Inc., Tulsa, Oklahoma).

Results

VDR RFLPs

No significant departures from Hardy-Weinberg equilibrium were observed for *Apa*I, Bsm I, and *Taq*I RFLPs. The distribution of genotypes was similar to that of other studies, in Caucasians. When the 3 RFLPs at the VDR gene locus were considered together in all subjects analyzed, 7 major genotypes were recognized: AaBbTt (n = 157), AABBtt (n = 71), aabbTT (n = 56). AABbTt (n = 49), AabbTT (n = 47), AAbbTT (n = 17), and AaBbTT (n = 13).

Clinical characteristics of patients, in relation to the VDR genotype, are shown in Table 2. Sixteen women having rare genotypes (n < 5) were excluded from analysis. Results indicated that subjects in the seven most common genotypes were well matched for age and did not significantly differ for YSM, height, weight, and dietary calcium intake. After correcting for potential confounding factors, a statistically significant segregation of VDR genotypes with lumbar BMD was detected ($P = 0.01$, analysis of covariance), with mean corrected BMD values at the lumbar spine significantly higher in women with aabbTT genotype, compared with those with either AABBtt or AaBbTt genotype ($P < 0.05$, Tukey's test). A similar, but not significant, trend was observed for femoral neck BMD, with a 6% higher BMD value in aabbTT than in AABBtt genotype (data not shown).

When Bsm I and *Taq*I polymorphisms were analyzed separately, we observed a significantly increased lumbar BMD in women with the bb or the TT genotypes than in those with BB and tt genotypes, respectively (0.87 ± 0.01 *vs.* 0.78 ± 0.02, bb *vs.* BB, $P < 0.05$, Tukey's test; and 0.86 ± 0.01 *vs.* 0.78 ± 0.02, TT *vs.* tt, $P < 0.05$, Tukey's test). Conversely, no differences in mean BMD levels for different *Apa*I genotypes were detected (data not shown).

Genotype determinations for osteoporotic, osteopenic, and normal groups are summarized in Table 3. We observed a significantly increased prevalence of AABBtt genotype in osteoporotic and osteopenic patients, compared with nonosteoporotic ($\chi^2 = 14.7$; $P = 0.0006$). On the contrary, aabbTT and AabbTT genotypes were significantly overrepresented in nonosteoporotic *vs.* osteoporotic women ($\chi^2 = 8.75$, df = 1, $P = 0.003$ for aabbTT genotype; and $\chi^2 = 6.05$, df = 1, $P = 0.04$ for AabbTT genotype). Cross-tabulation testing resulted statistically significant ($\chi^2 = 41.9$, df = 14, $P = 0.0004$).

ER RFLPs

*Pvu*II and *Xba*I RFLPs allele frequencies in this Italian population followed Hardy-Weinberg equilibrium. When we combined the 2 RFLPs we recognized six genotypes: PpXx (n = 174), ppxx (n = 126), PPXX (n = 73), Ppxx (n = 25), PPXx (n = 17), and PpXX (n = 11). Three genotypes (PPxx, ppXX, and ppXx) were not detected in the population examined in this study.

The clinical characteristics, by ER genotype, of the 426 studied women are given in Table 4. There were no significant differences in age, weight, height, YSM, and dietary calcium intake across genotypes. Analysis of the ER genotypes, in relation to adjusted BMD values, did not reveal any significant effect.

Single RFLP analysis revealed a trend for a 0.05 g/cm^2 higher lumbar BMD value in women with pp genotype than in those with PP genotype (data not shown). On the contrary, women with the xx genotype showed a 0.02 g/cm^2 lower lumbar BMD than those with XX genotype (data not shown). However, none of these differences reached statistical significance.

χ^2 analysis on the genotypic frequencies revealed no significant increased prevalence of any of the six ER genotypes

TABLE 3. VDR Genotypes for normal, osteopenic, and osteoporotic patients

Genotype	Patients			χ^2	*P* value
	Normal	Osteopenic	Osteoporotic		
AABBtt	11 (7.6%)	29 (18.9%)	40 (23,8%)	14.7	0.0006
AABbTt	15 (10.4%)	11 (10.4%)	23 (13.7%)	1.0	0.6
AaBbTt	56 (38.9%)	37 (34.9%)	64 (38.1%)	0.5	0.7
AAbbTT	8 (5.6%)	4 (3.8%)	5 (3.0%)	1.3	0.5
AaBbTT	5 (3.5%)	3 (2.8%)	5 (3.0%)	0.1	0.9
AabbTT	23 (16%)	12 (11.3%)	12 (7.1%)	6.0	0.04
aabbTT	26 (18%)	19 (17.9%)	11 (6.5%)	11.3	0.003

TABLE 2. Clinical features of the women in relation to VDR genotype; 16 of the 426 women exhibiting rare genotypes (n < 5) were excluded from analysis

	Genotype						
	AABBtt	AABbTt	AaBbTt	AAbbTT	AaBbTT	AabbTT	aabbTT
n	71	49	157	17	13	47	56
Age	55.8 ± 1.0	58.9 ± 1.4	57.4 ± 0.7	59.0 ± 2.7	58.8 ± 2.7	56.8 ± 1.3	57.2 ± 1.1
YSM	8.3 ± 1.6	9.7 ± 1.6	9.6 ± 0.8	7.9 ± 3.0	10.5 ± 3.3	9.8 ± 2.2	9.0 ± 1.4
Height (cm)	160.6 ± 0.9	161.5 ± 1.2	160.4 ± 0.7	163.6 ± 2.6	163.8 ± 2.6	163.1 ± 1.2	162.3 ± 1.0
Weight (kg)	63.4 ± 1.5	64.6 ± 2.0	63.2 ± 1.3	62.6 ± 4.1	63.1 ± 4.2	63.6 ± 1.9	65.0 ± 1.7
Ca intake (mg/day)	588 ± 162	607 ± 186	565 ± 132	549 ± 221	578 ± 227	551 ± 196	560 ± 189
FOA score	0.54 ± 0.12	0.70 ± 0.14	0.60 ± 0.08	0.47 ± 0.22	0.74 ± 0.30	0.63 ± 0.14	0.65 ± 0.12
SPO score	0.76 ± 0.12	0.61 ± 0.15	0.63 ± 0.09	0.58 ± 0.21	0.81 ± 0.27	0.70 ± 0.15	0.75 ± 0.13
Lumbar BMD	0.81 ± 0.03[a]	0.84 ± 0.03	0.85 ± 0.02[a]	0.87 ± 0.06	0.83 ± 0.07	0.89 ± 0.03	0.91 ± 0.03
Corrected BMD[b]	0.78 ± 0.02[a]	0.85 ± 0.03	0.82 ± 0.01[a]	0.85 ± 0.06	0.80 ± 0.07	0.87 ± 0.03	0.89 ± 0.02

Values are expressed as mean ± SEM.
[a] $P < 0.05$ Tukey's test *vs.* aabbTT genotype.
[b] Lumbar BMD values, corrected for age, height, weight, YSM, FOA, and SPO scores.

TABLE 4. Clinical features of the 426 women in relation to ER genotype

	Genotype					
	PPXX	PPXx	PpXX	PpXx	Ppxx	ppxx
n	73	17	11	174	25	126
Age	56.5 ± 1.0	58.2 ± 1.9	59.0 ± 2.6	57.6 ± 0.7	59.0 ± 1.7	58.1 ± 0.8
YSM	8.1 ± 1.2	9.5 ± 2.3	10.3 ± 3.1	9.3 ± 0.8	10.2 ± 2.0	9.7 ± 1.0
Height (cm)	161.5 ± 1.1	161.2 ± 2.3	163.7 ± 2.9	162.4 ± 0.7	158.3 ± 1.8	160.4 ± 0.8
Weight (kg)	62.6 ± 1.7	60.5 ± 3,2	65.4 ± 4.1	63.7 ± 1.1	63.3 ± 2.9	64.7 ± 1.3
Ca intake (mg/day)	554 ± 168	593 ± 221	556 ± 202	566 ± 121	601 ± 198	574 ± 138
FOA score	0.58 ± 0.12	0.49 ± 0.24	0.80 ± 0.30	0.43 ± 0.10	0.91 ± 0.18	0.76 ± 0.12
SPO score	0.85 ± 0.13	0.85 ± 0.27	0.90 ± 0.32	0.59 ± 0.10	0.83 ± 0.20	0.69 ± 0.11
Lumbar BMD	0.856 ± 0.02	0.872 ± 0.04	0.890 ± 0.04	0.850 ± 0.01	0.861 ± 0.03	0.873 ± 0.01
Corrected BMD[a]	0.825 ± 0.02	0.844 ± 0.04	0.866 ± 0.06	0.821 ± 0.01	0.833 ± 0.05	0.855 ± 0.01

Values are espressed as mean ± SEM.
[a] Lumbar spine BMD values, corrected for age, height, weight, YSM, SPO, and FOA scores.

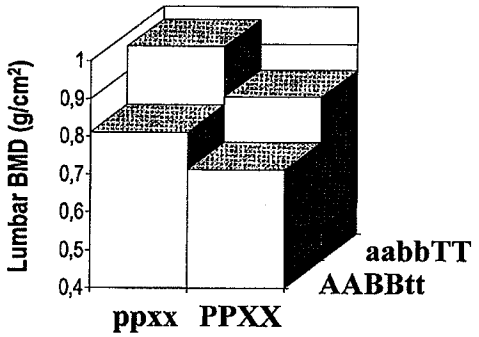

FIG. 1. Mean corrected lumbar BMD values, adjusted for potential confounding factors among women grouped by opposite VDR (AABBtt, aabbTT) and ER (PPXX, ppxx) genotypes.

in osteoporotic, osteopenic and nonosteoporotic groups (data not shown).

Combined effect of VDR and ER genes RFLPs

Sixteen major association groups were detected combining VDR and ER genotypes: AABBtt-PPXX (n = 9), AABBtt-PpXx (n = 29), AABBtt-ppxx(n = 25), AaBbTt-PPXX (n = 31), AaBbTt-PpXx (n = 70), AaBbTt-ppxx (n = 43), aabbTT-PPXX (n = 8), aabbTT-PpXx (n = 27), aabbTT-ppxx (n = 14), AaBbTt-Ppxx (n = 10), AABbTt-PPXX (n = 14), AABbTt-ppxx (n = 12), AABbTt-PpXx (n = 18), AabbTT-PPXX (n = 12), AabbTT-ppxx (n = 8), and AabbTT-PpXx (n = 23).

As shown in Fig. 1, when mean adjusted lumbar BMD values were calculated among women grouped by ER and VDR genotypes, we observed a statistically significant difference of approximately 0.26 g/cm^2 between the two opposite association AABBtt-PPXX and aabbTT-ppxx (0.713 ± 0.05 *vs.* 0.970 ± 0.03 g/cm^2, $P < 0.05$, Tukey's test). Furthermore, a trend for higher BMD values was detected between ppxx and PPXX subjects with the same VDR genotype ($P = 0.08$, AABBtt-PPXX *vs.* AABBtt-ppxx; $P = 0.07$, aabbTT-PPXX *vs.* aabbTT-ppxx).

Discussion

Evidence from epidemiological and twin studies clearly demonstrates that osteoporosis is a multifactorial disease with a strong genetic component. BMD, the major factor determining bone strength and consequently osteoporotic fracture risk, can be considered a quantitative polygenic trait. Because the description that the genetic component responsible for bone mass could be largely ascribed to a simple allelic change in the VDR gene (5), several conflicting reports on the relationship between VDR genotypes and BMD have been published (6–18, 22, 30). The VDR gene, however, is only one of a large group of candidate genes, ranging from those encoding cytokines and growth factors involved in local control of bone metabolism to those encoding collagenic and noncollagenic matrix components and those encoding calciotropic hormones and their receptors. Therefore, even though the VDR gene could represent a major gene in the determination of bone mass, other intrinsic and environmental influences certainly interact with it, and these interactions need to be quantified, to understand more of the causal spectrum (31).

Present data from a large and ethnically homogeneous population showed a significant segregation of VDR genotypes with lumbar BMD, in the Italian population, as previously shown in Australian women (5–6). Women in the AABBtt genotype group showed a spinal BMD 13% less than those with aabbTT genotype. Such a difference persisted even after adjustment of BMD values for the potential influence of SPO and FOA. The magnitude of this effect between extreme homozygotes was approximately 0.1 g/cm^2, slightly lower than that reported by Morrison *et al.* (5–6). A similar trend was observed for femoral neck BMD, but with no statistically significant difference between genotypes. How can we reconcile these evidences with the lack of segregation of VDR alleles with BMD in several patient samples (13–17)? One possible cause of discrepancy could be related to the relatively small samples of many other studies being not powerful enough to adequately assess the VDR gene allele effect on bone mass. This explanation does not apply to two recent studies that have looked specifically at the relationship between VDR genotypes and BMD in large samples of postmenopausal women (17, 30). The first study (30), of Uitterlinden and co-workers, reported a weak association between low bone mass and a particular VDR genotype, AAbbTT, which is different from the one reported to be associated with low BMD, by our and other groups (4–12). In the 426 analyzed Italian women, individuals with AAbbTT genotype showed BMD values that were interme-

diate, between those with aabbTT and AABBtt genotypes. However, Uitterlinden *et al.* limited their analysis to the femoral neck BMD, a site with predominantly cortical bone, whereas the genetic effect on bone mass seems to be stronger at sites with higher proportions of trabecular bone (2–5). In the second study (17), no significant relationships between VDR genotypes and BMD (measured at the spine, hip, and forearm) were detected in 268 French postmenopausal women, with frequency distribution of VDR genotypes quite different from that previously reported both by the same authors (14) and in other Caucasian populations of European ancestry (30, 32). Moreover, the contribution of VDR polymorphisms, both in Dutch and French studies, may have been masked by the relatively high calcium intake (17, 30). Indeed, environmental factors, such as calcium intake, are known to differ widely between populations and have been shown to contribute to the genetic influence of VDR genotypes on BMD (10–11), rates of bone loss (10–11), and intestinal calcium absorption (22). Consistent with the work of Dawson-Hughes *et al.* (22), we recently observed a significantly reduced fractional absorption capacity of strontium in a group of calcium-depleted women with AABBtt genotype, with respect to those with aabbTT genotype, suggesting a segregation of the VDR AABBtt genotype with a lower intestinal calcium absorption efficiency (32). For all these reasons, it is possible that the influence of VDR genotypes on bone metabolism could be observed only among populations with a relatively low calcium intake, as in the Italian population (33). Interestingly, in this sample of Italian postmenopausal women, we also observed a statistically significant increase in prevalence of both AABBtt genotype in the osteoporotic group and aabbTT and AabbTT genotypes in the nonosteoporotic group, in agreement with a possible segregation of the ABt homozygous haplotype with a higher risk of developing osteoporosis. Similarly, 2 other studies showed a disproportionate representation of the B allele in osteoporotic subjects, compared with a control group; but in both of them, these differences did not reach statistical significance (12, 18). By contrast, Looney and co-workers referred no large overrepresentation of the BB genotype in a group of North American severely osteoporotic women, compared with age-matched controls (15). However, all these previous studies were conducted in a limited number of subjects, with a consequent limited statistical power to test the hypothesis of a prevalence of a given genotype in osteoporotic subjects (12, 15, 16, 18).

The present study extends observations relating VDR genotypes and demonstrates that the addition of ER genotype to VDR genotype determination may provide a tool to identify, more precisely, individuals with a reduced bone mass. The magnitude of the effect of combining both VDR and ER genotype determination on lumbar BMD reaches approximately 2 SD and is significantly greater than that obtained from the analysis of the single ER or VDR gene effect. From this study, it also seems that the ER genotype PPXX confers some reduction in spinal BMD only when combined with VDR AABBtt genotype, without showing a segregation with BMD values by itself. Kobayashi *et al.* recently reported a statistically significant association of ER Px haplotype with a lower BMD in postmenopausal Japanese women, independent of VDR genotype (19). It is possible that these differences are related to the relative differential distribution of VDR and ER genotypes between populations of European and Asiatic ancestry. In fact, the PPxx genotype, representative of 8% of the Japanese population, was not detected in any of the 426 women examined in this study, whereas VDR genotype AABBtt, which in our Italian population is associated with the lowest BMD values, is extremely rare in the Japanese population (8, 16, 34). For this reason, a hypothetical segregation of BMD with polymorphisms at the ER gene locus could be more easily detectable in Asiatic women (where just 2 VDR genotypes, AabbTT and aabbTT, account for almost 80% of the total population) than in Caucasian populations of European ancestry, which exhibit high heterogeneity in VDR gene polymorphisms.

The epistatic effect between the ER and the VDR genes on BMD determination may be biologically fundamental, supporting a relevant role of the ER gene locus on BMD. Analysis of larger sample populations will make it possible to ascribe the ER gene locus either to the major gene family or to the polygenic aggregate, whose members are recognized among loci whose genetic effect is individually small. This approach, however, may be considered useful in future complex models of segregation analysis for osteoporotic risk.

In conclusion, in this homogeneous population of Italian postmenopausal women with a relatively low calcium intake, the allelic changes at the VDR gene locus are responsible for an important portion of the genetic component of spinal BMD. The results from the association analysis of ER and VDR genotypes effect on lumbar BMD suggest that the ER RFLPs could play a role, as well, exerting an additional contribution to bone mass determination. Other polymorphic genes and environmental factors could further modulate the expression of ER and VDR allelic effect on bone mass, making the picture as complicated as it is in nature.

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Erratum

In the announcement of drugs newly approved by the FDA on page 360 in the February issue of *The Journal of Clinical Endocrinology and Metabolism* [83(2):360], two entries were mistakenly conflated. The announcement should have read:

Repaglinide, trade name Prandin, made by Novonordisk.
Sibutramine, trade name Meridia, made by Knoll.
Raloxifene, trade name Evista, made by Lilly.

The Editors regret the confusion caused by this error.

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Biochemistry

Cloning and expression of full-length cDNA encoding human vitamin D receptor

(1α,25-dihydroxyvitamin D_3)

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ABSTRACT **Complementary DNA clones encoding the human vitamin D receptor have been isolated from human intestine and T47D cell cDNA libraries. The nucleotide sequence of the 4605-base pair (bp) cDNA includes a noncoding leader sequence of 115 bp, a 1281-bp open reading frame, and 3209 bp of 3′ noncoding sequence. Two polyadenylylation signals, AATAAA, are present 25 and 70 bp upstream of the poly(A) tail, respectively. RNA blot hybridization indicates a single mRNA species of ≈4600 bp. Transfection of the cloned sequences into COS-1 cells results in the production of a single receptor species indistinguishable from the native receptor. Sequence comparisons demonstrate that the vitamin D receptor belongs to the steroid- receptor gene family and is closest in size and sequence to another member of this family, the thyroid hormone receptor.**

Vitamin D_3 receptors (VDR) mediate the action of their cognate ligand 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] by controlling the expression of hormone-sensitive genes (1). These receptor proteins are intracellular polypeptides of 50–60 kDa that specifically bind 1,25$(OH)_2D_3$ and interact with target-cell nuclei to produce a variety of biologic effects (2, 3). Recently, monoclonal antibodies (mAbs) have been used to recover cDNAs encoding a portion of the chicken VDR mRNA (4). The present study describes the cloning and characterization of cDNAs encoding the human vitamin D receptor (hVDR).¶ Analysis of the deduced amino acid sequence of hVDR demonstrates that this protein belongs to the superfamily of trans-acting transcriptional regulatory factors defined by the steroid and thyroid hormone receptors (5–9). These receptors are characterized by a highly conserved DNA-binding domain rich in cysteine, lysine, and arginine residues and a carboxyl-terminal hydrophobic ligand-binding domain. The primary sequence of the hVDR exhibits this characteristic domain structure and shows that the VDR is more closely related to the thyroid hormone receptor than to the other receptors. Expression of the cloned cDNA in COS-1 cells demonstrates that the single protein produced is indistinguishable from the native receptor in both physical properties and affinity for 1,25$(OH)_2D_3$.

MATERIALS AND METHODS

Identification of hVDR cDNAs. An oligo(dT)-primed λgt10 cDNA library (10) made from 5 μg of human jejunal poly(A)$^+$ mRNA was screened using a 310-base pair (bp) *Eco*RI–*Sau*3A fragment of cVDR (4). The filters were hybridized at 42°C overnight in the following solution: 5× SSC (1× SSC = 150 mM sodium chloride and 15 mM trisodium citrate)/5× Denhardt's solution (11)/0.1% sodium dodecyl sulfate/salmon sperm DNA at 200 μg·ml^{-1}/50% (wt/vol) formamide containing 1 × 10^6 cpm of nick-translated probe per ml (12). After being washed three times at 60°C in 0.5× SSC to remove excess probe, the filters were exposed to X-ray film (Kodak X-Omat S) at −70°C with an intensifying screen. Hybridization-positive phage were isolated, and their inserts were subcloned into the *Eco*RI site of M13mp8. λVDR1 was obtained in this fashion and subsequently used to screen an Okayama–Berg (13) T47D cDNA library (provided by G. Ringold, Stanford University), yielding clone VDR3, and a specifically primed λgt10 T47D library yielding clone λVDR2. The latter was made by substituting the oligonucleotide 5′ ACACACCCCACAGATCCGGGG 3′ for oligo(dT) in the first strand reaction (underlined in Fig. 2).

DNA Sequence Analysis. Three overlapping clones were used to generate the full-length VDR sequence cDNA inserts to be sequenced. These clones were subcloned into the *Eco*RI site of M13mp8 for sequencing by the dideoxynucleotide chain-termination method (14). Primers were either the M13 universal primer or sequence-derived oligonucleotides.

RNA Blot Hybridization. Total RNA was isolated from each of three cell lines (15), and the mRNA fraction was selected by successive passages over oligo(dT)-cellulose (16). The mRNA samples (10 μg) were resolved on a 1% formaldehyde–agarose gel (17) and then transferred electrophoretically to a nylon membrane (Nytran; Schleicher & Schuell). The filter was hybridized to nick-translated hVDR-1 (1 × 10^8 cpm/μg; 1 × 10^6 cpm/ml) using the conditions described above.

Expression of Recombinant hVDR in COS-1 Cells. Two cDNA clones, λVDR1 and VDR3, were joined at a common restriction site (*Stu* I) and cloned into the expression vector P91023B (18) recreating the full-length coding sequence for hVDR. COS-1 cells were transfected with 12 μg of expression plasmid DNA per 100-mm plate using DEAE-dextran (19). After 48 hr, cytosols from 2 × 10^7 cells were prepared by Dounce homogenization in 10 mM Tris·HCl, pH 7.6/0.3 M KCl/5 mM dithiothreitol (designated TK0.3D buffer). Cytosols were diluted to 4 × 10^5 cell equivalents per ml, and 0.1-ml aliquots were incubated with increasing concentrations of 1α,25-dihydroxy[26,27-*methyl*-^{3}H]D_3 {1,25$(OH)_2$[^{3}H]D_3} (176 Ci/mmol, Amersham; 1 Ci = 37 GBq) with or without a 100-fold molar excess of unlabeled 1,25$(OH)_2D_3$. After incu-

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Abbreviations: VDR, vitamin D_3 receptors; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; hVDR, human VDR; 1,25$(OH)_2$[^{3}H]D_3, 1α,25$(OH)_2$[26,27-*methyl*-^{3}H]D_3; mAb, monoclonal antibody.

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¶The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Intelligenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03258).

bation at 4°C for 20 hr, specific binding was determined by hydroxyapatite binding assay (20). Similarly, other D metabolites (25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, and 1,24,25-trihydroxyvitamin D_3, courtesy of M. Uskokovic, Hoffmann–La Roche) were used to compete for $1,25(OH)_2D_3$ in this assay to determine the binding preference for expressed VDR.

Sucrose Gradient Sedimentation. COS-1 cell cytosols were prepared 48 hr posttransfection, as described above, and then incubated for 4 hr at 4°C with 1 nM $1,25(OH)_2D_3$ with or without a mAb to cVDR (mAb 9A7γ). A parallel incubation was done without the mAb but with a 100-fold molar excess of unlabeled $1,25(OH)_2D_3$. Samples were layered onto 4.6-ml gradients of 10–30% sucrose prepared in TK0.3D buffer and centrifuged at 265,000 × *g* for 18 hr at 4°C. Gradients were fractionated from the top and quantitated in ACS scintillation cocktail (Amersham).

RESULTS

Identification of VDR cDNA Clones. Two mAbs were used to isolate a putative cVDR cDNA clone from a chick intestinal λgt11 cDNA expression library (as reported in ref. 4). The deduced amino acid sequence of this original clone contained a cysteine-, lysine-, and arginine-rich domain. This, together with hybrid-selected translation data and the pattern of mRNA regulation, provided preliminary evidence that this clone encoded the chicken VDR. We have used this 310-bp fragment of cVDR cDNA to isolate the corresponding full-length human cDNA.

An oligo(dT)-primed λgt10 cDNA library was constructed from 5 μg of human intestinal (jejunum) poly(A)$^+$ mRNA. A single hybridizing clone (clone λVDR1) was obtained from this library of 500,000 primary recombinants. After being subcloned into M13mp8, the sequence of the 1100-bp fragment was determined by the dideoxynucleotide chain-termination method of Sanger (14). The 5′ 576 bp of the insert cloned in λVDR1 is highly similar to the corresponding chicken sequence, but thereafter the two sequences completely diverge, and there is no longer an open reading frame in the sequence from clone λVDR1. The presence of a consensus splice sequence at the point of divergence suggests that clone λVDR1 sequence might represent a partially spliced mRNA. Therefore, the λVDR1 sequence was used to screen an Okayama–Berg cDNA library made from T47D cell mRNA (a human breast cancer cell line). Two positive clones, out of 2 × 10^6 primary recombinants, were obtained and characterized by restriction mapping. The largest of these, clone VDR3, contains an insert of 4.3 kb that overlaps with clone λVDR1 to give a 4.5-kb cDNA sequence, close to the predicted size of hVDR mRNA from RNA analysis (Fig. 1). The encoded open reading frame begins after the *Eco*RI linker sequences and extends for 427 amino acids until a TAG stop codon. To confirm the sequences around the linker and to obtain further 5′ sequence, a specifically primed cDNA library was prepared from T47D mRNA using a 17-mer complementary to a region close to the 5′ end of λVDR1. Four independent clones isolated from this library confirmed and extended the 5′ terminal sequence. A restriction map of the composite cDNA clones is shown in Fig. 1.

Sequence of Human VDR mRNA and Protein. Fig. 2 shows the nucleotide and deduced amino acid sequence of the full-length human vitamin D receptor mRNA. The overlapping cDNA clones λVDR1, VDR3, and λVDR2 comprise 4605 bp and contain a noncoding leader sequence of 115 bp, a 1281-bp open reading frame, and 3209 bp of 3′ noncoding sequence. The functional significance of such a long 3′ untranslated region is unknown, although this is a common feature of all the steroid receptors cloned to date. The sequence AATAAA, thought to be required for polyadenylylation, is represented twice, 25 bp and 70 bp upstream from the poly(A) tract. This

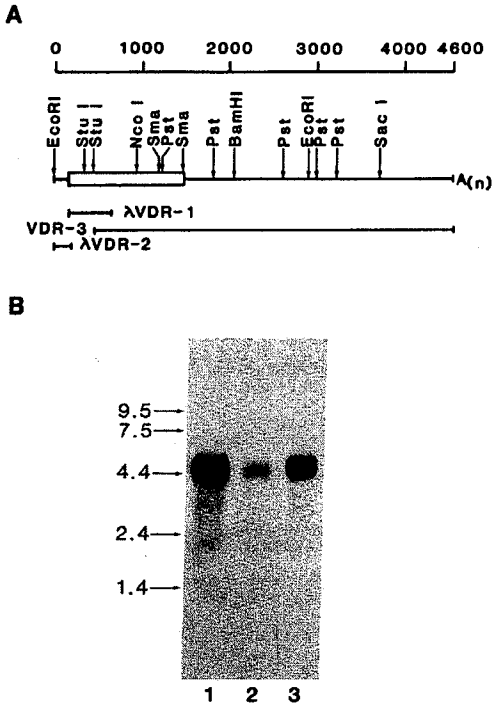

FIG. 1. (*A*) Restriction map of the overlapping hVDR cDNA clones. The boxed region indicates the coding sequence. (*B*) RNA blot hybridization analysis of hVDR transcripts from cell line T47D (lane 1), human intestine ATCC cell line 407 (lane 2), and cell line HL-60 (lane 3) poly(A)$^+$ mRNAs.

is consistent with the RNA data (Fig. 1) in which the predominant mRNA species is ≈4.6 kilobases (kb).

The predicted hVDR translation product is 427 amino acids long with a calculated M_r of 48,295; this agrees well with published data (21, 22) if post-translational modifications are considered. For example, the VDR is known to be phosphorylated (23). Assignment of the initiating methionine was from the general observation that translation of most eukaryotic mRNAs begins at the 5′ proximal AUG (24). Definitive demonstration of this initiation site awaits amino-terminal amino acid sequencing of purified receptor protein.

Identification of the functional domains of hVDR may help establish the mechanisms involved in receptor-mediated transcriptional activation. Comparison of the coding sequence of hVDR with other steroid receptors (5–7, 9) and thyroid hormone receptor (8) shows significant regions of similarity (Fig. 3). The area of strongest identity (C1 in Fig. 3) is an ≈70-amino acid hydrophilic domain that is rich in cysteine, lysine, and arginine residues and lies towards the amino terminus of VDR. Importantly, the position of each of the nine cysteines is conserved among these different proteins, suggesting a common functional domain. This region has been proposed to mediate the DNA binding properties of the steroid receptors (25). Deletion analysis of the glucocorticoid receptor and estrogen receptor has confirmed that this C1 domain is involved in both DNA binding and transcriptional activation (26–28). Preliminary results show that this is also true for the vitamin D receptor (unpublished work). The arrangement of the conserved cysteines led Weinberger *et al.* (25) to suggest that this region can form a Zn^{2+}-dependent "finger" structure analogous to that proposed for the 5S transcription factor TF111A (29). No definitive evidence for this hypothesis exists at present. A second region of lesser conservation, which we have termed C2, lies towards the carboxyl terminus of the VDR protein and is separated from

```
-115 GGAACAGCTTGTCCACCCGCCGGCCGGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGACCTCACAGAAGAGCACCCCTGGGCTCCAC

 -25 TTACCTGCCCCCTGCTCCTTCAGGGATGGAGGCAATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCG
                                MetGluAlaMetAlaAlaSerThrSerLeuProAspProGlyAspPheAspArgAsnValProAr
                                                                                10                  20
  65 GATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAAGGCTGCAAAGGCTTCTTCAGGCGAAGCAT
     gIleCysGlyValCysGlyAspArgAlaThrGlyPheHisPheAsnAlaMetThrCysGluGlyCysLysGlyPhePheArgArgSerMe
                            30                  40                  50
 155 GAAGCGGAAGGCACTATTCACCTGCCCCTTCAACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAA
     tLysArgLysAlaLeuPheThrCysProPheAsnGlyAspCysArgIleThrLysAspAsnArgArgHisCysGlnAlaCysArgLeuLy
                            60                  70                  80
 245 ACGCTGTGTGGACATCGGCATGATGAAGGAGTTCATTCTGACAGATGAGGAAGTGCAGAGGAAGCGGGAGATGATCCTGAAGCGGAAGGA
     sArgCysValAspIleGlyMetMetLysGluPheIleLeuThrAspGluGluValGlnArgLysArgGluMetIleLeuLysArgLysGl
                            90                  100                 110
 335 GGAGGAGGCCTTGAAGGACAGTCTGCGGCCCAAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCCACCATAAGAC
     uGluGluAlaLeuLysAspSerLeuArgProLysLeuSerGluGluGlnGlnArgIleIleAlaIleLeuLeuAspAlaHisHisLysTh
                            120                 130                 140
 425 CTACGACCCCACCTACTCCGACTTCTGCCAGTTCCGGCCTCCAGTTCGTGTGAATGATGGTGGAGGGAGCCATCCTTCCAGGCCCAACTC
     rTyrAspProThrTyrSerAspPheCysGlnPheArgProProValArgValAsnAspGlyGlySerHisProSerArgProAsnSe
                            150                 160                 170
 515 CAGACACACTCCCAGCTTCTCTGGGGACTCCTCCTCCTCCTGCTCAGATCACTGTATCACCTCTTCAGACATGATGGACTCGTCCAGCTT
     rArgHisThrProSerPheSerGlyAspSerSerSerSerCysSerAspHisCysIleThrSerSerAspMetMetAspSerSerSerPh
                            180                 190                 200
 605 CTCCAATCTGGATCTGAGTGAAGAAGATTCAGATGACCCTTCTGTGACCCTAGAGCTGTCCCAGCTCTCCATGCTGCCCCACCTGGCTGA
     eSerAsnLeuAspLeuSerGluGluAspSerAspAspProSerValThrLeuGluLeuSerGlnLeuSerMetLeuProHisLeuAlaAs
                            210                 220                 230
 695 CCTGGTCAGTTACAGCATCCAAAAGGTCATTGGCTTTGCTAAGATGATACCAGGATTCAGAGACCTCACCTCTGAGGACCAGATCGTACT
     pLeuValSerTyrSerIleGlnLysValIleGlyPheAlaLysMetIleProGlyPheArgAspLeuThrSerGluAspGlnIleValLe
                            240                 250                 260
 785 GCTGAAGTCAAGTGCCATTGAGGTCATCATGTTGCGCTCCAATGAGTCCTTCACCATGGACGACATGTCCTGGACCTGTGGCAACCAAGA
     uLeuLysSerSerAlaIleGluValIleMetLeuArgSerAsnGluSerPheThrMetAspAspMetSerTrpThrCysGlyAsnGlnAs
                            270                 280                 290
 875 CTACAAGTACCGCGTCAGTGACGTGACCAAAGCCGGACACAGCCTGGAGCTGATTGAGCCCCTCATCAAGTTCCAGGTGGGACTGAAGAA
     pTyrLysTyrArgValSerAspValThrLysAlaGlyHisSerLeuGluLeuIleGluProLeuIleLysPheGlnValGlyLeuLysLy
                            300                 310                 320
 965 GCTGAACTTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGATCGTCCTGGGGTGCAGGACGCCGCGCTGAT
     sLeuAsnLeuHisGluGluGluHisValLeuLeuMetAlaIleCysIleValSerProAspArgProGlyValGlnAspAlaAlaLeuIl
                            330                 340                 350
1055 TGAGGCCATCCAGGACCGCCTGTCCAACACACTGCAGACGTACATCCGCTGCCGCCACCCGCCCCCGGGCAGCCACCTGCTCTATGCCAA
     eGluAlaIleGlnAspArgLeuSerAsnThrLeuGlnThrTyrIleArgCysArgHisProProProGlySerHisLeuLeuTyrAlaLy
                            360                 370                 380
1145 GATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGCTGCCTCTCCTTCCAGCCTGAGTGCAGCAT
     sMetIleGlnLysLeuAlaAspLeuArgSerLeuAsnGluGluHisSerLysGlnTyrArgCysLeuSerPheGlnProGluCysSerMe
                            390                 400                 410
1235 GAAGCTAACGCCCCTTGTGCTCGAAGTGTTTGGCAATGAGATCTCCTGACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCC
     tLysLeuThrProLeuValLeuGluValPheGlyAsnGluIleSerEnd
                            420
1325 AGGGCCACGTGCCAGGCCCGGGGCTGGCGGCTACTCAGCAGCCCTCCTCACCCGTCTGGGGTTCAGCCCCTCCTCTGCCACCTCCCCTAT
1415 CCACCCAGCCCATTCTCTCTCCTGTCCAACCTAACCCCTTTCCTGCGGGCTTTTCCCCGGTCCCTTGAGACCTCAGCCATGAGGAGTTGC
1505 TGTTTGTTTGACAAAGAAACCCAAGTGGGGGCAGAGGGCAGAGGCTGGAGGCAGGCCTTGCCCAGAGATGCCTCCACCGCTGCCTAAGTG
1595 GCTGCTGACTGATGTTGAGGGAACAGACAGGAGAAATGCATCCATTCCTCAGGGACAGAGACACCTGCACCTCCCCCCACTGCAGGCCCC
1685 GCTTGTCCAGCGCCTAGTGGGGTCTCCCTCTCCTGCCTTACTCACGATAAATAATCGGCCCACAGCTCCCACCCCACCCCCTTCAGTGCC
1775 CACCAACATCCCATTGCCCTGGTTATATTCTCACGGGCAGTAGCTGTGGTGAGGTGGGTTTTCTTCCCATCACTGGAGCACCAGGCACGA
1865 ACCCACCTGCTGAGAGACCCAAGGAGGAAAAACAGACAAAAACAGCCTCACAGAAGAATATGACAGCTGTCCCTGTCACCAAGCTCACAG
1955 TTCCTCGCCCTGGGTCTAAGGGGTTGGTTGAGGTGGAAGCCCTCCTTCCACGGATCCATGTAGCAGGACTGAATTGTCCCCAGTTTGCAG
2045 AAAAGCACCTGCCGACCTCGTCCTCCCCCTGCCAGTGCCTTACCTCCTGCCCAGGAGAGCCAGCCCTCCCTGTCCTCCTCGGATCACCGA
2135 GAGTAGCCGAGAGCCTGCTCCCCCACCCCCTCCCCAGGGGAGAGGGTCTGGAGAAGCAGTGAGCCGCATCTTCTCCATCTGGCAGGGTGG
2225 GATGGAGGAGAAGAATTTTCAGACCCCAGCGGCTGAGTCATGATCTCCCTGCCGCCTCAATGTGGTTGCAAGGCCGCTGTTCACCACAGG
2315 GCTAAGAGCTAGGCTGCCGCACCCCAGAGTGTGGGAAGGGAGAGCGGGGCAGTCTCGGGTGGCTAGTCAGAGAGAGTGTTTGGGGGTTCC
2405 GTGATGTAGGGTAAGGTGCCTTCTTATTCTCACTCCACCACCCAAAAGTCAAAAGGTGCCTGTGAGGCAGGGGCGGAGTGATACAACTTC
2495 AAGTGCATGCTCTCTGCAGGTCGAGCCCAGCCCAGCTGGTGGGAAGCGTCTGTCCGTTTACTCCAAGGTGGGTCTTTGTGAGAGTGAGCT
2585 GTAGGTGTGCGGGACCGGTACAGAAAGGCGTTCTTCGAGGTGGATCACAGAGGCTTCTTCAGATCAATGCTTGAGTTTGGAATCGGCCGC
2675 ATTCCCTGAGTCACCAGGAATGTTAAAGTCAGTGGGAACGTGACTGCCCCAACTCCTGGAAGCTGTGTCCTTGCACCTGCATCCGTAGTT
2765 CCCTGAAAACCCAGAGAGGAATCAGACTTCACACTGCAAGAGCCTTGGTGTCCACCTGGCCCCATGTCTCTCAGAATTCTTCAGGTGGAA
2855 AAACATCTGAAAGCCACGTTCCTTACTGCAGAATAGCATATATATCGCTTAATCTTAAATTTATTAGATATGAGTTGTTTTCAGACTCAG
2945 ACTCCATTTGTATTATAGTCTAATATACAGGGTAGCAGGTACCACTGATTTGGAGATATTTATGGGGGGAGAACTTACATTGTGAAACTT
3035 CTGTACATTAATTATTATTGCTGTTGTTATTTTACAAGGGTCTAGGGAGAGACCCTTGTTTGATTTTAGCTGCAGAACTGTATTGGTCCA
3125 GCTTGCTCTTCAGTGGGAGAAAAACACTTGTAAGTTGCTAAACGAGTCAATCCCCTCATTCAGGAAAACTGACAGAGGAGGGCGTGACTC
3215 ACCCAAGCCATATATAACTAGCTAGAAGTGGGCCAGGACAGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCAGTTTGGGAGGTCGA
3305 GGTAGGTGGATCACCTGAGGTCGGGAGTTCGAGACCAACCTGACCAACATGGAGAAACCCTGTCTCTATTAAAAATACAAAAAAAAAAAA
3395 AAAAAAAAATAGCCGGGCATGGTGGCGCAAGCCTGTAATCCCAGCTACTCAGGAGGCTGAGGCAGAAGAATTGAACCCAGGAGGTGGAGG
3485 TTGCAGTGAGCTGAGATCGTGCCGTTACTCTCCAACCTGGACAACAAGAGCGAAACTCCGTCTTAGAAGTGGACCAGGACAGGACCAGAT
3575 TTTGGAGTCATGGTCCGGTGTCCTTTTCACTACACCATGTTTGAGCTCAGACCCCCACTCTCATTCCCCAGGTGGCTGACCCAGTCCCTG
3665 GGGGAAGCCCTGGATTTCAGAAAGAGCCAAGTCTGGATCTGGGACCCTTTCCTTCCTTCCCTGGCTTGTAACTCCACCAAGCCCATCAGA
3755 AGGAGAAGGAAGGAGACTCACCTCTGCCTCAATGTGAATCAGACCCTACCCCACCACGATGTGCCCTGGCTGCTGGGCTCTCCACCTCAG
3845 GCCTTGGATAATGCTGTTGCCTCATCTATAACATGCATTTGTCTTTGTAATGTCACCACCTTCCCAGCTCTCCCTCTGGCCCTGCTTCTT
3935 CGGGGAACTCCTGAAATATCAGTTACTCCAGCCCTGGGCCCCACCACCTAGGCCACTCCTCCAAAGGAAGTCTAGGAGCTGGGAGGAAAAG
4025 AAAAGAGGGGAAAATGAGTTTTTATGGGGCTGAACGGGGAGAAAAGGTCATCATCGATTCTACTTTAGAATGAGAGTGTGAAATAGACAT
4115 TTGTAAATGTAAAACTTTTAAGGTATATCATTATAACTGAAGGAGAAGGTGCCCCAAAATGCAAGATTTTCCACAAGATTCCCAGAGACA
4205 GGAAAATCCTCTGGCTGGCTAACTGGAAGCATGTAGGAGAATCCAAGCGAGGTCAACAGAGAAGGCAGGAATGTGTGGCAGATTTAGTGA
4295 AAGCTAGAGATATGGCAGCGAAAGGATGTAAACAGTGCCTGCTGAATGATTTCCAAAGAGAAAAAAAGTTTGCCAGAAGTTTGTCAAGTC
4385 AACCAATGTAGAAAGCTTTGCTTATGGTAATAAAAATGGCTCATACTTATATAGCACTTACTTTGTTTGCAAGTACTGCTGTAAATAAAT
4475 GCTTTATGCAAACCAAAAAAAAAAAAAAAAAAAAAA  4510
```

FIG. 2. Nucleotide and deduced amino acid sequence of full-length hVDR cDNA. Both sequences are numbered with respect to the putative initiating methionine. The oligonucleotide used to generate the specifically primed cDNA library is underlined.

the DNA binding domain by an intervening nonconserved "hinge" of ≈150 amino acids. This domain represents the approximate amino-terminal limits of the ligand-binding domain, based on mutational analysis of glucocorticoid and estrogen receptors (26–28). A third domain, C3, which is further toward the carboxyl terminus of the molecule is quite similar to the equivalent region in the thyroid hormone receptor based on either amino acid- or nucleotide-sequence alignments (Fig. 3 *B* and *C*) but not similar to the other receptors. This suggests a structural and/or evolutionary relationship between these two receptors and between their respective ligands.

Expression of Functional VDR Receptor. To demonstrate that the cloned sequences can direct the synthesis of a functional receptor protein, two of the cDNAs (λVDR1 and VDR3) were joined at a common restriction site and cloned

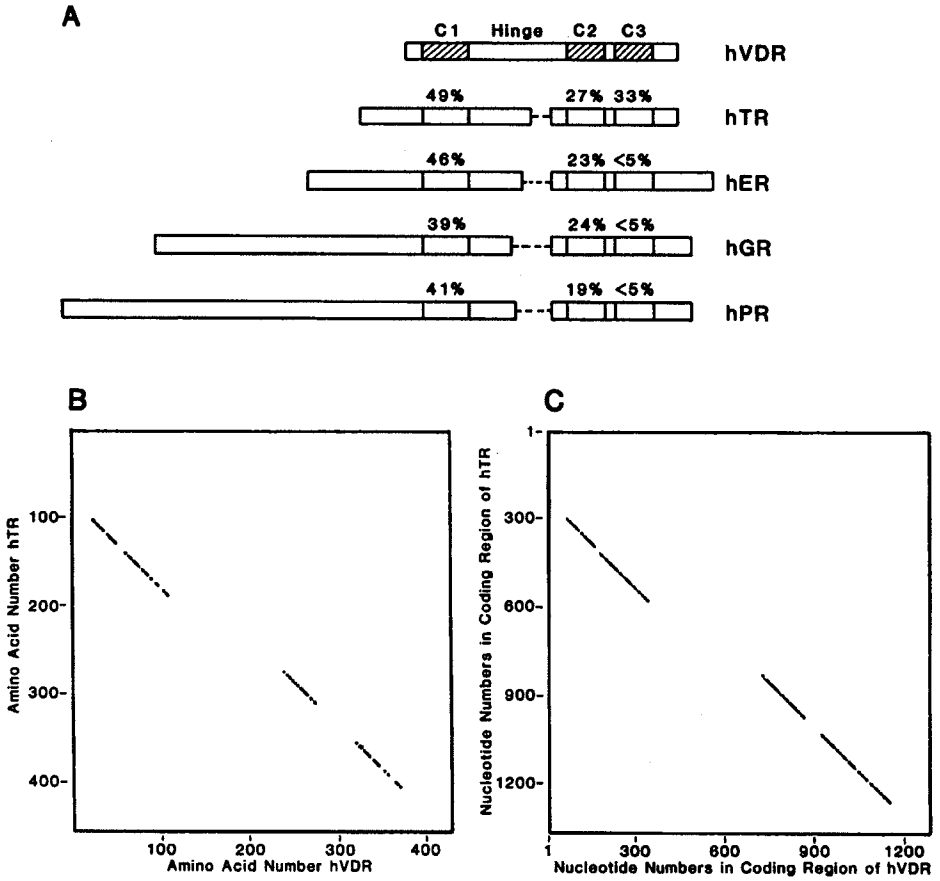

FIG. 3. (*A*) Schematic comparison between the deduced amino acid sequences of hVDR, human glucocorticoid (hGR), human estrogen (hER), human thyroid hormone (hTR), and human progesterone (hPR) receptors (5–9). Sequences were aligned for maximum similarity about their DNA binding (C1) or C2 domains. Percent similarities indicated are with respect to hVDR sequence. (*B* and *C*) Dot matrix plot of the regions of nucleotide- and amino acid-sequence similarity between hVDR and human thyroid hormone receptor.

into the expression vector P91023B in both orientations. This vector uses the adenovirus major late promoter and simian virus 40 origin of replication upstream of the cloned sequences with the mouse dihydrofolate reductase coding region and simian virus 40 polyadenylylation signal downstream (18). The final expression constructs, phVDR1 and phVDR2, contain 2.1 kb of hVDR sequence in either the correct or inverted orientation with respect to the promoter. These two plasmids were used to transfect COS-1 monkey kidney cells, and the resulting transient pools were analyzed for receptor expression.

A competitive binding assay was used to assess the hormone binding properties of the expressed protein. Cytosolic extracts were incubated with $1,25(OH)_2[^3H]D_3$ with or without a 100-fold molar excess of unlabeled steroid, and receptor–hormone complexes were measured using a hydroxylapatite binding assay (20). The phVDR1-transfected cells exhibit substantial $1,25(OH)_2D_3$-binding activity, which is saturable at 1 nM. Scatchard analysis (Fig. 4*A*) indicates the expression of $\approx$220,000 copies of hVDR per cell with a K_d of 5.2×10^{-11} M. The binding affinity of the steroid for the recombinant hVDR is identical to that reported for vitamin D receptors from a number of sources (3, 30), although the level of receptor expression is some 20-fold higher than that found in receptor-rich cell lines such as 3T6 (30) and T47D (31). The results in Fig. 4*B* illustrate that recombinant hVDR displays selectivity for $1,25(OH)_2D_3$ over such metabolites as 25-hydroxyvitamin D_3, 24(R),25-dihydroxyvitamin D_3, 1α,24(R),25-trihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, and vitamin D_3. The resulting rank order of receptor preference for individual vitamin D metabolites is identical to that of wild-type receptor in all tissues and cells examined to date (31, 32).

Lastly, sedimentation of $1,25(OH)_2[^3H]D_3$-labeled extracts on 10–30% hypertonic sucrose gradients revealed a receptor species sedimenting at 3.2S. Preincubation with a mAb to cVDR shifted this peak to 7S (Fig. 4*C*); both peaks are completely displaceable with excess unlabeled $1,25(OH)_2D_3$. These results are consistent with those found for native hVDR (33).

DISCUSSION

In this paper we have presented the complete nucleotide sequence of the cDNA for hVDR. Expression of hVDR cDNA in COS-1 cells confirms that the protein encoded by the cDNA represents the authentic receptor on the following criteria. (*i*) The protein binds $1,25(OH)_2D_3$ with the same affinity as does the native receptor. (*ii*) The expressed protein exhibits the same rank order binding preferences for other vitamin D metabolites. (*iii*) It sediments on sucrose gradients at 3.2S and displays the characteristic shift to 7S with receptor antibody.

$1,25(OH)_2D_3$ is thought to act via this receptor to effect changes in gene expression, much like the steroid receptors. However, this has been difficult to confirm because the VDR is very low in abundance and few VDR-regulated genes have been identified. The nucleotide sequence data and the deduced amino acid sequence show that considerable similarity exists among VDR, the steroid hormone receptors, and the thyroid hormone receptor. In particular, a cysteine-, lysine-, and arginine-rich domain is $\approx$40% conserved between VDR, thyroid receptor, and the steroid receptors. This domain seems capable of forming a Zn^{2+}-dependent "finger" and probably harbors both DNA binding and transcriptional activities of the receptors. The presence of this common structural motif in the VDR argues that the mechanism of vitamin D action truly parallels that of the other hormone systems. Furthermore, it has been suggested that VDR and thyroid hormone receptor are closely related biochemically, based

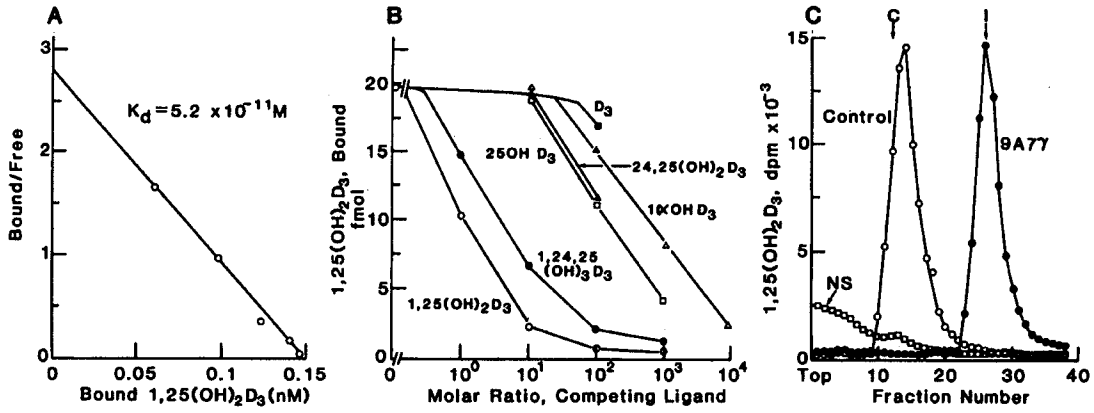

FIG. 4. Expression of hVDR cDNA in transiently transfected COS-1 cells. (*A*) Saturation analysis of recombinant hVDR in phVDR1-transfected COS-1 cells. The Scatchard analysis shows that the expressed receptor has a K_d of 5.2×10^{-11} M and a B_{max} of 2.2×10^5 sites per cell. (*B*) Vitamin D_3 metabolite specificity of hVDR. Transfected COS-1 cell cytosol was incubated with 1,25(OH)$_2$[^{3}H]D$_3$ and the indicated molar concentrations of 25-hydroxy-, 24,25-dihydroxy-, 1α-hydroxy-, 1α,24,25-trihydroxy-vitamin D_3, or vitamin D_3 for 18 hr at 4°C followed by assay using hydroxyapatite (18). (*C*) Sedimentation analysis of recombinant hVDR. Cytosol was prepared 48 hr after transfection and incubated for 4 hr at 4°C with 1,25(OH)$_2$[^{3}H]D$_3$ (1 nM) with (●) or without (○) 9A7 cVDR mAb. A similar incubation was done with 1,25(OH)$_2$[^{3}H]D$_3$, but this was done with a 100-fold molar excess of 1,25(OH)$_2$D$_3$ and without the mAb (□). C, cytosol with labeled D$_3$; I, cytosol plus mAb with labeled D$_3$; NS, cytosol with excess unlabeled D$_3$.

largely on their strong nuclear binding characteristics (34). The overall sequence similarities presented here support a close evolutionary relationship between these two receptors.

Steroid receptors interact with enhancer-like sequences usually found in the 5′-flanking DNA of regulated genes (35). However, little is known about the sequences involved in the presumed genomic action of the VDR. The availability of cloned receptor sequences will expedite studies of vitamin D-responsive regulatory elements. In addition, expression of truncated receptors (unpublished work) and examination of aberrant receptors found in vitamin D-dependent rickets type II (36) will help to define functional domains of the receptor molecule.

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Structural Organization of the Human Vitamin D Receptor Chromosomal Gene and Its Promoter

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The vitamin D receptor (VDR) is known to mediate the pleiotropic biological actions of 1,25-dihydroxyvitamin D_3 through its ability to modulate the expression of target genes. The regulation of this ligand-activated cellular transcription factor is reported to occur at both transcriptional and post-translational levels. To begin to address the molecular basis by which the VDR gene is regulated transcriptionally, we report here an initial characterization of the human VDR gene and its promoter. We isolated several overlapping λ-phage and cosmid clones that cover more than 100 kb of human DNA and contained the entire VDR gene. The gene is comprised of 11 exons that, together with intervening introns, span approximately 75 kb. The noncoding 5′-end of the gene includes exons 1A, 1B, and 1C. Eight additional exons (exons 2–9) encode the structural portion of the VDR gene product. While primer extension and S1 nuclease-mapping studies reveal several common transcriptional start sites, three unique mRNA species are produced as a result of the differential splicing of exons 1B and 1C. The DNA sequence lying upstream of exon 1A is GC rich and does not contain an apparent TATA box. Several potential binding sites for the transcription factor SP1 and other activators are evident. Fusion of DNA fragments containing putative promoter sequences upstream of the luciferase structural gene followed by transient transfection of these plasmids into several mammalian cell lines resulted in significant reporter activity. Due to the size and complexity of the 5′-end of the VDR gene, we examined the activity of a DNA fragment surrounding exon 1C. An intron fragment 3′ of exon 1C conferred retinoic acid responsivity when fused to a reporter gene plasmid, suggesting a molecular mechanism for the previously observed ability of retinoic acid to induce the VDR. The recovery of the gene for the human VDR will enable further studies on the transcriptional regulation of this gene. (Molecular Endocrinology 11: 1165–1179, 1997)

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INTRODUCTION

The biological actions of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] that regulate transcriptional events within the nucleus of target cells are mediated by the vitamin D receptor (VDR)(1–3). This ligand-activated transcription factor belongs to a superfamily of genes that encode receptors for the steroid, thyroid, and vitamin A (retinoic acid and 9 *cis*- retinoic acid) hormones as well as orphan receptor proteins for which ligands have not yet been identified (4–6). Most members of this receptor gene family have undergone considerable scrutiny during the past several years, and significant progress has been made in understanding their structure and function. Although genes that encode proteins involved in mineral metabolism are a principal focus of VDR action and its 1,25-$(OH)_2D_3$ ligand *in vivo* (7, 8), the receptor is also capable of influencing genes whose products regulate more fundamental processes of cellular proliferation and differentiation (9, 10). The extensive biological effects now ascribed to 1,25-$(OH)_2D_3$ can be attributed, in large part, to the nearly ubiquitous tissue distribution of the VDR as well as to the highly selective regulation of

gene expression by this hormone in specific cell types (1).

Many of the chromosomal genes for the nuclear receptor family members have been cloned and their structural organization determined (11–15). These genes are often more than 50 kb in length and are comprised of multiple protein domain-associated exons separated by exceedingly large introns. An exception to this appears to be the orphan receptors, chicken ovalbumin upstream promoter-transcription factors α and β, which are encoded by genes that span 4–6 kb of DNA, contain only two introns, and thus may represent ancestral members of this superfamily of genes (16). The promoter regions of several of the receptor genes have been characterized. They often appear to resemble housekeeping genes, and many are embedded in GC-rich islands (11, 13). The absence of a TATA box is a common feature of several of these genes as is the existence of multiple start sites for transcription. In some cases, mRNA transcripts are produced from more than one promoter (17, 18); in others, transcripts are differentially spliced to create unique mRNA species that encode functionally distinct receptor isoforms (19, 20). Thus, nuclear receptor genes can be typified by the general structural organization of the encoding exons as well as through the overall characteristics of many of their promoters.

Transcriptional regulation of nuclear receptor gene expression is clearly evident. While receptors for metabolites of vitamin D and vitamin A, as well as the glucocorticoids are widely expressed (21), receptors for estrogen, progesterone, and androgens display a more restricted tissue-specific expression profile (22). Determinants of restricted expression of these genes in tissues and cells as well as the mechanisms that lead to activation of otherwise silent receptor genes during development or during the differentiation of specific cell types are generally not known. Expressed genes are, however, regulated by a number of nonpeptides hormones as well as peptide hormones, growth factors, and cytokines. Transcriptional autoregulation of receptor genes by their respective gene products through *cis* elements localized adjacent to cognate promoters is not uncommon. The β-retinoic acid receptor gene (RARβ), for example, is autoregulated through a β-retinoic acid response element located immediately adjacent to its promoter (23, 24) and accounts for substantial up-regulation of RARβ by retinoic acid both *in vivo* and *in vitro* (25, 26).

The VDR is regulated at both transcriptional and posttranslational levels. VDR gene expression is up-regulated in NIH-3T3 mouse fibroblasts through activation of the protein kinase A pathway (27) and down-regulated through activation of the protein kinase C pathway (28). Both of these signal transduction pathways mediate the actions of PTH on target cells (29) and thus may represent homeostatic mechanisms that ultimately control cellular sensitivity to 1,25-$(OH)_2D_3$. Other growth factors and cytokines are also known to regulate VDR gene expression, possibly through the above pathways. Estrogens (30), thyroid hormone (31), glucocorticoids (32), and retinoic acid (33, 34) are likewise able to alter VDR mRNA levels in what appear to be tissue-specific patterns of expression. Interestingly, both cell cycle (35) and the differentiation state of cells in culture (36) influence the extent of VDR mRNA expression. The level of the VDR under these circumstances may play a regulatory role in the control of cellular proliferation and differentiation by the vitamin D hormone in a wide variety of cell types. Finally, homologous up-regulation of VDR mRNA by 1,25-$(OH)_2D_3$ has also been demonstrated both *in vitro* and *in vivo*, again in a very tissue-specific fashion (37–39). Whether this regulatory action occurs directly as a result of VDR interaction with a *cis*-element(s) at the 5′-end of the VDR gene, analogous to the interaction of RARs with the RARβ gene promoter, or indirectly as a result of the induction or activation of other transcription factors remains to be demonstrated.

To gain a better understanding of the molecular mechanisms by which the VDR is regulated at the transcriptional level, we cloned the chromosomal gene for the human VDR. We report here on structural characterization and preliminary functional activity of its promoter. The single gene for human VDR spans more than 75 kb of genomic DNA and contains 11 exons. Three exons make up the 5′-noncoding region, and the eight additional exons encode the structural component of the VDR. The promoter is characterized by the lack of a TATA box initiator, its GC-rich nature, and the presence of putative binding sites for SP1 and a variety of transcription factors. It directs the transcription of at least three VDR mRNA transcripts in kidney that appear to arise from the differential splicing of 5′-noncoding exons. The promoter also directs the transcription of a chimeric gene when fused upstream of an expression plasmid containing the luciferase structural gene. The recovery of the human VDR gene and these initial studies provide the basis for further detailed examination of the transcriptional regulation of the VDR gene. This effort is particularly warranted in view of the central role of the VDR in 1,25-$(OH)_2D_3$ action and the potential therapeutic role of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs in blocking cellular proliferation and promoting the differentiation of tumor cells (40, 41).

RESULTS

The Structure and Sequence of hVDR mRNAs

We established first the authenticity of the 5′-end of the hVDR mRNA sequence reported initially by Baker *et al.* (42) using the 5′-RACE (rapid amplification of cDNA ends) PCR technique. Human kidney RNA was employed as template to prepare first-strand cDNA, and the latter was amplified using an anchored oligonucleotide (primer A) as well as a downstream primer (primer B2) corresponding to hVDR cDNA sequence as outlined in Fig. 1A. Primary amplification was fol-

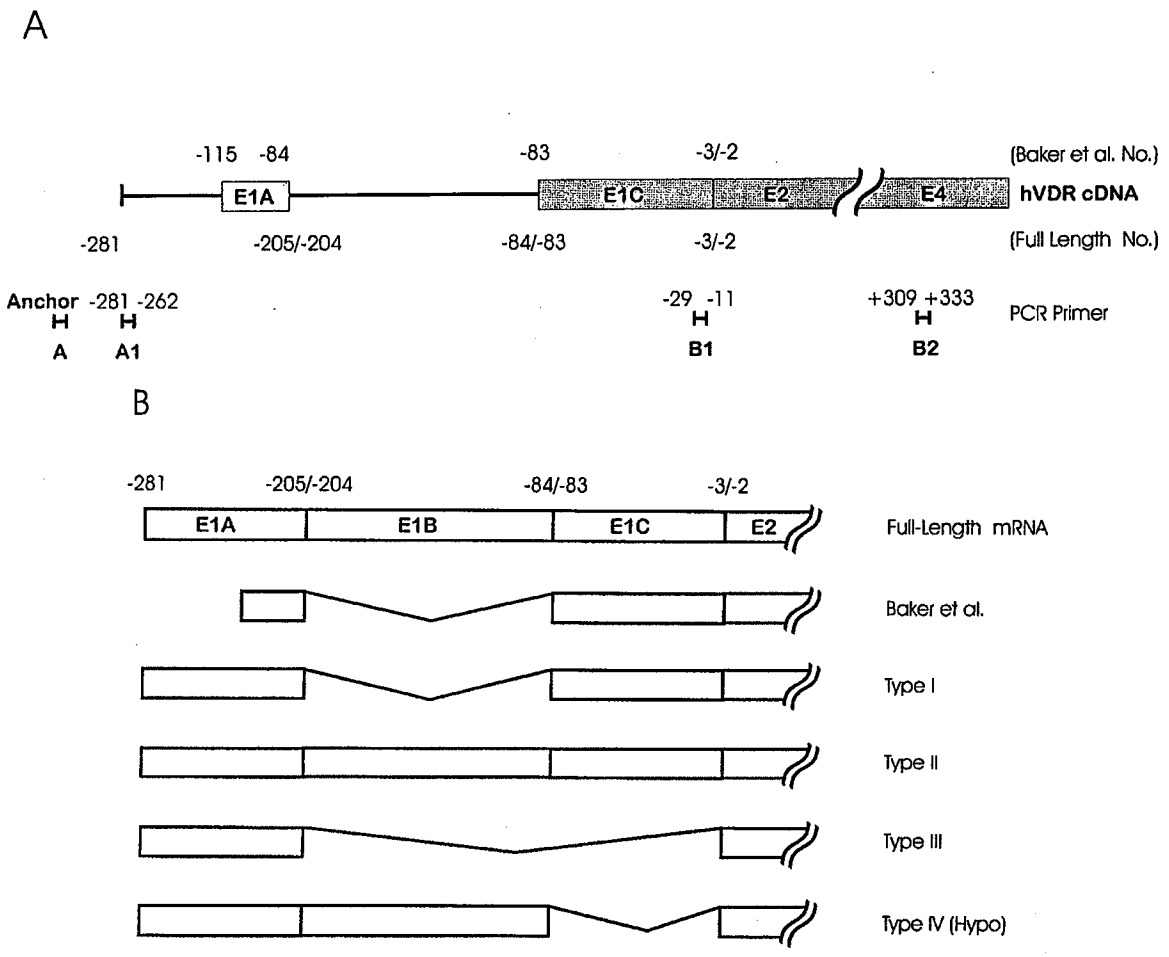

Fig. 1. Analysis of hVDR mRNA Transcripts Using 5′-RACE

A, Location of oligonucleotide primers used for DNA amplification. The *shaded rectangle* illustrates portions of the hVDR transcript originally reported by Baker *et al.* (42), whereas the *single line* indicates additional portions of the hVDR mRNA transcript revealed through 5′-RACE. The *numbering above* the diagram corresponds to that of Baker *et al.* (42) whereas the *numbering below* corresponds to the largest hVDR mRNA transcript that was identified. The location of DNA primers used to characterize the hVDR mRNA transcripts by 5′-RACE are indicated relative to the largest unspliced hVDR mRNA transcript identified. The anchor primer (corresponding to vector sequence) was obtained from Life Sciences. B, The three hVDR mRNA transcripts identified through the 5′-RACE technique are illustrated (type I, II, and III) relative to the transcript identified by Baker *et al.* (42). Type I and type III transcripts contain deletions that correspond to proposed exon 1B (type I) and exons 1B and 1C (type III), respectively. The type III transcript contains all the proposed exons: 1A, 1B, and 1C. Exon 2 (43) is common to all transcripts. The type IV transcript is hypothetical. The illustration is numbered relative to the full-length transcript (type II).

lowed by a secondary amplification as described in *Materials and Methods* using primers A and B1. The cloning and DNA sequence analysis of the resulting products revealed two individual transcripts. As observed in Table 1, the sequence of the first (type 1) was identical to that reported by Baker *et al.* (42) with the exception that the sequence of the DNA product extended some 45 bp upstream. This suggests that the original cDNA sequence reported by Baker *et al.* (42) was incomplete at the 5′-end by 45 bases and that the type 1 PCR product might represent the authentic 5′-end of the hVDR mRNA. The second transcript (type 2) was identical to the type 1 transcript (see Table 1) with the exception that a 121-bp insert was discovered lying between nucleotides −84 and −83 relative to the translation start site reported by Baker and colleagues (42). An additional amplification of kidney RNA was then carried out using primer A1 complementary to the authentic 5′-end of the mRNA established above and primer B2 as illustrated in Fig. 1A. Cloning and sequence analysis of the products of this reaction revealed the previous two mRNAs as well as a third (type 3). As documented in Table 1, type 3 was similar to type 1 with the exception that nts −83 to −3 relative to the sequence of Baker *et al.* (42) were deleted. The failure of primer B1 to hybridize to type 3 transcripts clearly prevented detection of type 3 in the original primary amplification described above. The

Table 1. Nucleotide Sequences of hVDR in RNA Transcripts Detected by 5′-RACE

Baker *et al*	5′-**GGAACAGCTT GTCCACCCGC CGGCCGGACC AGAAGCCTTT GGGTCTGAAG**	**+50**
	TGTCTGTGAG ACCTCACAGA AGAGCACCCC TGGGCTCCAC TTACCTGCCC	**+100**
	CCTGCTCCTT CAGGGATG-3′	**+150**
Type I	5′-CTGCTTGTCA AAAGGCGGCA GCGGAGCCGT GTGCGCCGGG AGCGC**GGAAC**	**+50**
	AGCTTGTCCA CCCGCCGGCC GGACCAGAAG CCTTTGGGTC TGAAGTGTCT	**+100**
	GTGAGACCTC ACAGAAGAGC ACCCCTGGGC TCCACTTACC TGCCCCCTGC	**+150**
	TCCTTCAGGG ATG-3′	**+200**
Type II	5′-CTGCTTGTCA AAAGGCGGCA GCGGAGCCGT GTGCGCCGGG AGCGC**GGAAC**	**+50**
	AGCTTGTCCA CCCGCCGGCC GGACCAGGCT CCTGAACCTA GCCCAGCTGG	**+100**
	ACGGAGAAAT GGACTCTAGC CTCCTCTGAT AGCCTCATGC CAGGCCCCGT	**+150**
	GCTCATTGCT TTGCTTGCCT CCCTCAATCC TCATAGCTTC TCTTTGGG**AA**	**+200**
	GCCTTTGGGT CTGAAGTGTC TGTGAGACCT CACAGAAGAG CACCCCTGGG	**+250**
	CTCCACTTAC CTGCCCCCTG CTCCTTCAGG GATG-3′	**+300**
Type III	5′-CTGCTTGTCA AAAGGCGGCA GCGGAGCCGT GTGCGCCGGG AGCGC**GGAAC**	**+50**
	AGCTTGTCCA CCCGCCGGCC GGACCAGGGA TG-3′	**+100**

The preexisting sequences documented by Baker *et al.* (42) are indicated in *bold*. Accession numbers for these sequences are AB002157–AB002168.

organization of the three transcripts are illustrated in Fig. 1B relative to the sequence of Baker *et al.* (42). The nature of these transcripts, one of which contains an insertion and the other a deletion, suggests that the 5′-end of the hVDR gene contains four exons, three of which are exclusively noncoding exons, and that at least two of these exons may undergo unique splicing events in the kidney. Based upon these data, a fourth unique transcript containing a deletion of exon 1C is possible.

λ-Phage Genomic Clones

Having characterized the 5′-end of the hVDR mRNA, we used the 5′-RACE-derived DNA fragments to screen a λ-phage genomic library and recovered several positive clones. Three clones were mapped using the restriction enzymes *Bam*HI, *Eco*RI, and *Sac*I, and relevant probe-reactive fragments were sequenced. As observed in Fig. 2, λ-clones 1 and 2 represent overlapping clones that contain exons 1A and 1B, which are in turn separated by an intron of approximately 5 kb. λ-Clone 3 did not overlap λ-clone 2 but contained exon 1C and exon 2. Exon 2 contained the start site of translation beginning 3 bp downstream of the 5′-end of the exon. The intron located between exons 1C and 2 was determined to be approximately 4 kb, but the lack of contiguity between λ-clones 2 and 3 prevented determination of the size of the intron located between 1B and 1C. The presence of these four exons is entirely consistent with the data obtained from analysis of the hVDR mRNA. We designated the first three exons 1A, 1B, and 1C to be consistent with a

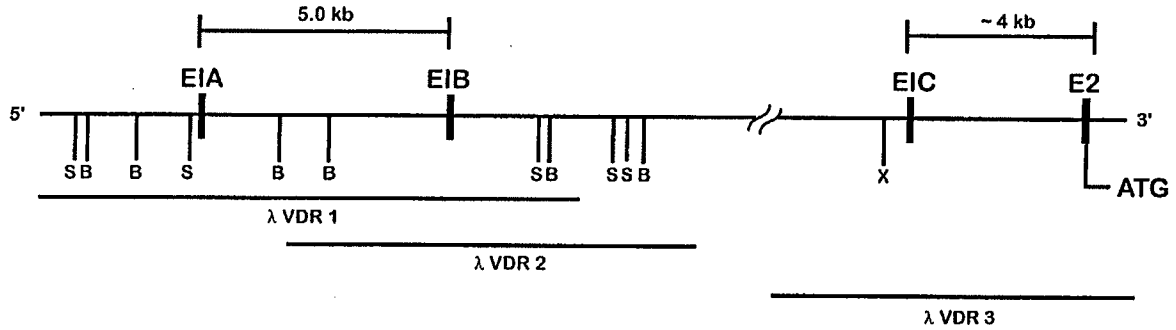

Fig. 2. Organizational Map of Exons 1A, 1B, 1C, and 2 Comprising the 5′- End of the hVDR Chromosomal Gene

A λ-phage genomic library was screened using 5′-RACE products to obtain three genomic fragments containing the hVDR gene. Each clone was restriction mapped and relevant portions sequenced. Restriction mapping and sequence analysis revealed that two overlapping clones (λ-VDR1 and λ-VDR2) contained sequence corresponding to proposed exons 1A and 1B (type II) separated by 5 kb (intron A). A downstream noncontiguous clone (λ-VDR3) contained exons IC and exon 2 (type II) separated by 4 kb (intron B). The distances between exons 1A and 1B and between exons 1C and 2 were determined by restriction mapping followed by Southern blot analysis. S, *Sac*I; X, *Xho*I; B, *Bam*HI. ATG represents the first translation codon.

preliminary description of the organization of this gene (43).

Cosmid Genomic Clones

In view of the lack of contiguity between λ-clones 2 and 3, and to recover the remainder of the hVDR gene, we screened a human liver genomic library prepared in the cosmid vector pCV109 (44) sequentially with several nick-translated hVDR cDNA probes. Screening resulted in the recovery of four unique individual human DNA cosmid clones designated phVDRG1, 3, 4, and 11. As illustrated in Fig. 3A, *Eco*RI, *Kpn*I, *Xho*I, and *Sal*I restriction endonucleases were used initially to map each of the respective clones and revealed that they represent overlapping fragments of 40 to 50 kb which together span approximately 93 kb of contiguous genomic DNA.

Three unique hVDR cDNA probes (see *Materials and Methods*) were used to determine the extreme 5′- and 3′-boundaries of the VDR gene within this locus and to determine the relative orientation of individual DNA restriction fragments located within these four clones with respect to the hVDR mRNA. Southern blot hybridization analysis with these respective VDR cDNA probes revealed that clones G1, G3, and G11 likely contained exons comprising the entire VDR chromosomal gene. Subsequent detailed hybridization analysis of G3 and G11 DNA digested with *Eco*RI, *Kpn*I, or *Eco*RI and *Kpn*I utilizing hVDR cDNA probe 3 (nt $^{+}10$ to $^{+}2012$ which includes the entire normal hVDR open reading frame) revealed the presence of eight hybridizable DNA fragments spanning a total of 55 kb (Fig. 3B, *left*). The locations of these fragments (identified as B4.2, C2.2, D4.4, E8.0, F5.5, G5.6, H2.7, and I7.0) in relationship to the genomic cosmid clones are depicted in Fig. 3A. Importantly, each of these DNA restriction fragments was unequivocally identified in human genomic DNA after high-stringency Southern blot hybridization analysis with a combination of the cDNA probes indicated above. Six of these fragments (C-H) are detected after analysis of *Eco*RI- and *Kpn*I-digested human DNA with probe 3 (Fig. 3B, *right*).

The position of exon 1A of 77 bases in genomic clone phVDRG1 was verified through hybridization screening with an oligonucleotide corresponding to a portion of exon 1A. DNA fragment A was sequenced to identify the first exon located as illustrated in Fig. 3A. Exons 1B (121 bases), 1C (81 bases), and 2 (148 bases) located in this clone were mapped relative to exon 1A, further defining the approximately 20 kb size of the intron located between exons 1B and 1C (see figure). DNA fragments B and C (see Fig. 3A) were cloned and sequenced to identify the intron/exon boundaries of exons 1C and 2. Exon 1B was positioned relative to exon 1A in λ-clone 1.

Additional DNA fragments identified through hybridization analysis were isolated, subcloned, and either partially or completely sequenced to determine the precise location of downstream VDR exons. As observed in Fig. 3C, the gene is split into seven additional exons (exons 3 through 9) of 131, 185, 121, 172, 152, 117, and 3466 bp, which, together with the upstream exons, form the authentic full-length hVDR gene. The gene itself, however, spans some 75 kb of DNA, the majority consisting of introns whose sizes, boundaries, and locations within the mature hVDR mRNA are documented in Table 2. A comparison of the sequences of the intron/exon boundaries indicates that each conforms to a canonical splice consensus sequence typical of most eukaryotic genes.

The relative organization of the exons and their locations with respect to domains located in the hVDR protein (45) is depicted in Fig. 3C. Exon 2 contains 2 bp of noncoding sequence, the translation initiation codon, and the N terminal of the two dissimilar DNA-binding Zn^{++} finger modules. Exon 3 lies downstream of a 15-kb intron and encodes the second DNA-binding zinc module. Exons 4–6 are clustered together; exons 4 and 5 encode a region that serves a hypothetical hinge function between DNA- and steroid-binding portions of the receptor protein, while exon 6 encodes the remainder of the hinge and the first portion of the steroid-binding domain. Finally, clustered exons 7–9 encode the C-terminal half of the VDR, with exon 9 containing the final 85 amino acids as well as the remaining approximately 3200 bp that constitute the large 3′-noncoding sequence.

An Exon 2 Translation Site Polymorphism

Exon sequences within the VDR gene derived here were compared with those of the human VDR cDNA recovered from the T47D breast cancer cell line and reported by Baker *et al.* (42). Exon sequences corresponded to those found in the hVDR cDNA with one exception, a T to C transition that eliminated the most 5′-ATG codon within the T47D VDR mRNA (see Table 2). The likely result of this mutation, which creates a potential polymorphic *Fok*I site within exon 2, is the utilization of a second in-frame translation codon beginning 10 nucleotides downstream that ultimately encodes a potentially foreshortened receptor protein of 424 amino acids. Evaluation of additional human DNA samples via DNA amplification techniques revealed that this feature of the cloned gene was not unique to the genomic library from which the hVDR gene was recovered (data not shown). More importantly, perhaps, the existence of an hVDR mRNA of the latter type has been demonstrated recently by Saijo *et al.* (46). Despite these observations, however, it will be necessary to confirm that two proteins that differ in molecular mass by only three amino acids are in fact translated in human tissues or cells.

The hVDR Promoter

We sequenced a DNA fragment extending 5′ of the start site of transcription containing the putative promoter for hVDR (Fig. 4). While this promoter lacks consensus TATA or CAAT boxes, the region is GC rich with five binding motifs for the transcription factor SP1 lying between

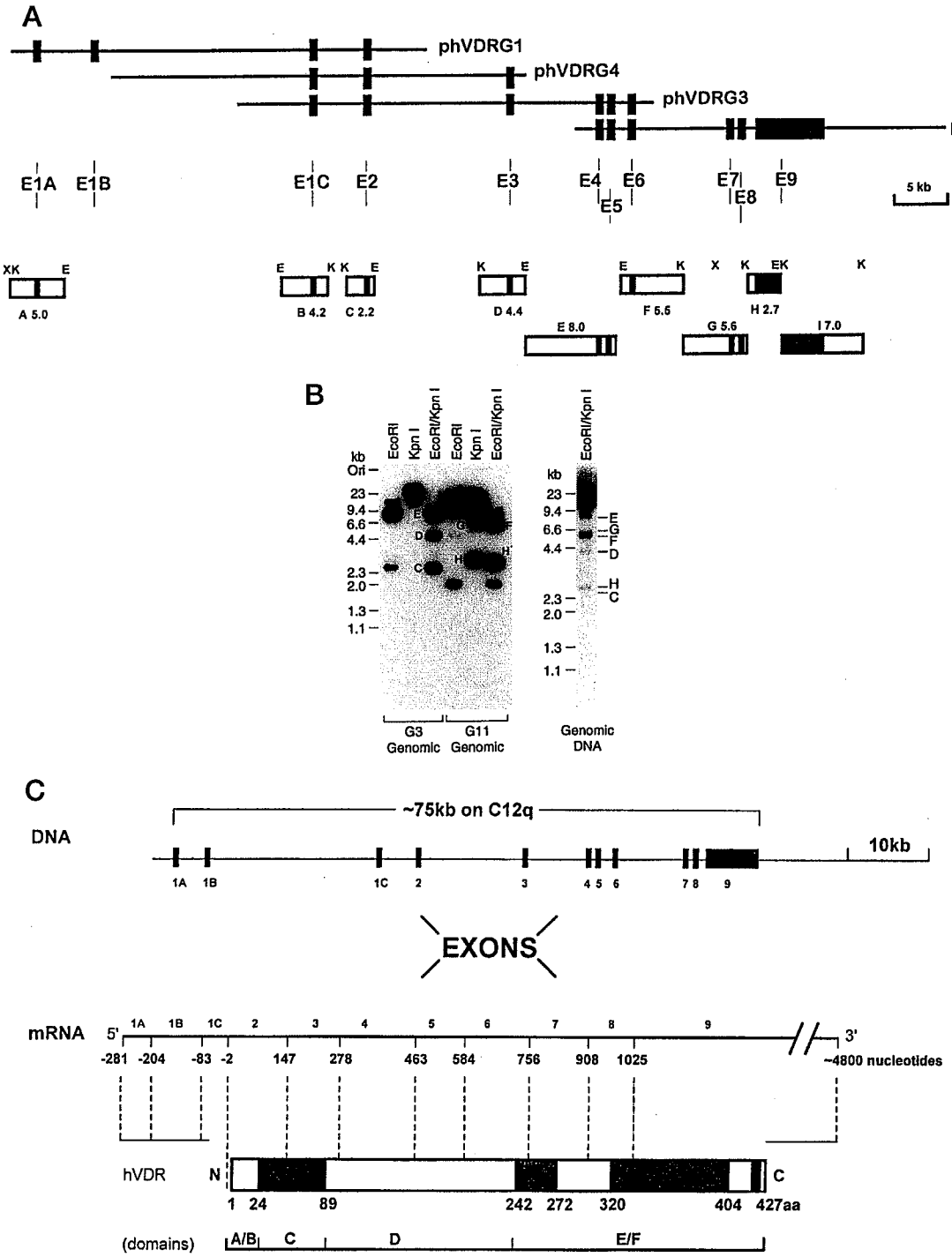

Fig. 3. Organizational Map of the Complete hVDR Chromosomal Gene

A, Organizational map of the complete hVDR chromosomal gene. A human liver cosmid library was screened with hVDR cDNA probes to obtain four clones (phVDRG1, phVDRG4, phVDRG3, and phVDRG11) that spanned the entire hVDR gene. Restriction mapping and Southern blot analysis revealed the overlapping nature of the clones as well as the relative position of each exon within the clone as indicated. Relevant restriction fragments (A5.0, B4.2, C2.2, D4.4, E8.0, F5.5, G5.6, H2.7, and I7.0) indicated as *rectangles* were subcloned and subjected to high resolution restriction mapping and sequencing. X, *Xho*I; K, *Kpn*I; E, *Eco*RI. The positions of the hVDR exons, which are numbered E1A, E1B, E1C, E2-E9, are indicated by the *black bars*. B, Southern blot analysis of phVDRG3 and phVDRG11 DNA and human genomic DNA. Overlapping phVDRG clones 3 and 11 were subjected to restriction digestion with *Eco*RI, *Kpn*I, or both enzymes as indicated (*left*). Human genomic DNA was subjected to simultaneous

Table 2. Features of the hVDR Gene

Exons			Exon/Intron Sequence		
No.	Size (bp)	Position	5′-Exon Boundary	Intron (Size, kb)	3′-Exon Boundary
1A	77	−281/−205	CGGACCAG**gt**gcgaaccc	· · · A (4.95) · · ·	tatccttaa**g**GCTCCTGAACCTAGC
1B	121	−204/−84	TCTTTGGG**gt**aagtacag	· · · B (~20) · · ·	ttcatttca**g**AAGCCTTTGGGTCTG
1C	81	−83/−3	TCCTTCAG**gt**aggtgttt	· · · C (~4) · · ·	gttcttaca**g**GG **ATG** GAG GCA **ATG** Met Glu Ala Met 1
2	148	−2/146	TTC AG**gt**gagccctc Phe Ar 49	· · · D (13.2) · · ·	ctctcggca**g**G CGA AGC ATG g Arg Ser Met 49
3	131	147/277	GAG T**gt**gagtgtcc Glu P 93	· · · E (8.4) · · ·	caccccaca**g**TC ATT CTG ACA he Ile Leu Thr 93
4	185	278/462	TTC CGG**gt**atgtctgc Phe Arg 154	· · · F (0.252) · · ·	tcccttcca**g**CCT CCA GTT CGT Pro Pro Val Arg 155
5	121	463/583	TCA G**gt**aagcagga Ser A 195	· · · G (1.32) · · ·	tctctccca**g**AC ATG ATG GAC sp Met Met Asp 195
6	172	584/755	TTC AG**gt**aagaaacc Phe Ar 252	· · · H (9.2) · · ·	tcccctgca**g**A GAC CTC ACC g Asp Leu Thr 252
7	152	756/907	AAA G**gt**atgcctag Lys A 303	· · · I (0.205) · · ·	tctctcaca**g**CC GGA CAC AGC la Gly His Ser 303
8	117	908/1024	CCA G**gt**atggggcc Pro A 342	· · · J (~1.4) · · ·	gtgcccaca**g**AT CGT CCT GGG sp Arg Pro Gly 342
9	3466	1025/3′ END			

The size and position of each of the 11 exons relative to the hVDR mRNA type II transcript are documented. The intron/exon boundaries as well as the approximate size of the introns are indicated. The canonical splice site consensus sequences are indicated in *bold*. The polymorphism associated with the two start sites of translation are *underlined* and shown in *bold*.

nucleotide (nt) −72 and −34 relative to the transcription start site (47). Potential binding sites for other transcription factors are also evident (48). In addition, five AGGTCA-like sequences, which represent potential nuclear receptor-binding element half-sites (49), are located between nt −1394 and −949. The functional relevance of these, as well as of additional interesting sequences, will need to be determined.

Activity of the 5′-Flanking Region of the hVDR Promoter

To evaluate the transcriptional capacity of the hVDR promoter, we cloned a series of 5′-deletion fragments of the gene into a luciferase reporter gene and transfected them individually into two mammalian cell lines. We tested constructs beginning at −1.935, −1.479, −1.221, −0.586, −0.464, −0.103, −0.034 kbp relative to the start site of transcription. The downstream boundary of each construct was located at the 3′-boundary of exon 1A at +71 relative to the start site of transcription [−89 relative to the start site of translation reported by Baker *et al.* (42)]. As observed in Fig. 5A, pVDE1–1.93 was capable of directing significant luciferase expression when introduced by transient transfection into HeLa cells. Each deletion construct likewise exhibited

digestion with *Kpn*I and *Eco*RI (*right*). Southern blot analysis was carried out as described in *Materials and Methods* and hybridized to the hVDR3 probe. Bands D-H identified both in genomic DNA and within the clones represent the DNA fragments schematically illustrated in A. Band C was weakly evident on the autoradiogram but not reproducible in the figure. C, Structural organization of the human chromosomal vitamin D receptor gene. The structural organization of the human VDR gene locus (DNA) comprising 11 exons (1A, 1B, 1C, 2 through 9) spanning approximately 75 kb of DNA is depicted. A 10 kb scale is indicated to the *right*. The location of exons relative to the mRNA transcript of ~4800 nucleotides (mRNA) and the encoded VDR protein of 427 amino acids (hVDR) is illustrated. With regard to the hVDR mRNA, negative numbers indicate 5′-noncoding nucleotides, and positive numbers indicate protein encoding nucleotides beginning with +1 indicated by Baker *et al.* (42) as well as 3′-untranslated sequences. Numbers *below* the hVDR protein indicate the amino acid residue boundaries of *shaded* homology domains. Regions of functionality are designated A/B, C, D, and E/F as described in Ref. 76.

Fig. 4. Sequence Determination of the hVDR Gene Promoter

The nucleotide sequence of the hVDR gene promoter is documented. The transcription start site is indicated as +1 beginning within the *boxed sequence*. The locations of potential binding sites for specific transcriptional regulators are *underlined* and indicated. Transcription factors indicated include SP-1, AP-1, AP-2, NFkB, GATA-1, Pit-1, and C/EBP. Potential binding sites for nuclear receptors are also present.

substantial transcriptional activity, although this activity varied and did not exhibit an obvious pattern. Constructs containing proximal elements of the promoter (−464 and −103 relative to the start site) displayed the most activity, suggesting the possibility that elements upstream of −0.464 kb may confer a negative regulatory function. 5′-Deletion of the hVDR promoter from −103 bp to −34 bp that removed four of the five GC boxes near the initiator resulted in a 10-fold drop in activity. The latter result substantiates the hypothesis that these elements play an important role in the activity of the hVDR promoter and provide the basis for future studies. Preliminary studies in Hela cells suggest that the 5′-flanking region of the gene is not responsive to 1,25-$(OH)_2D_3$ (data not shown).

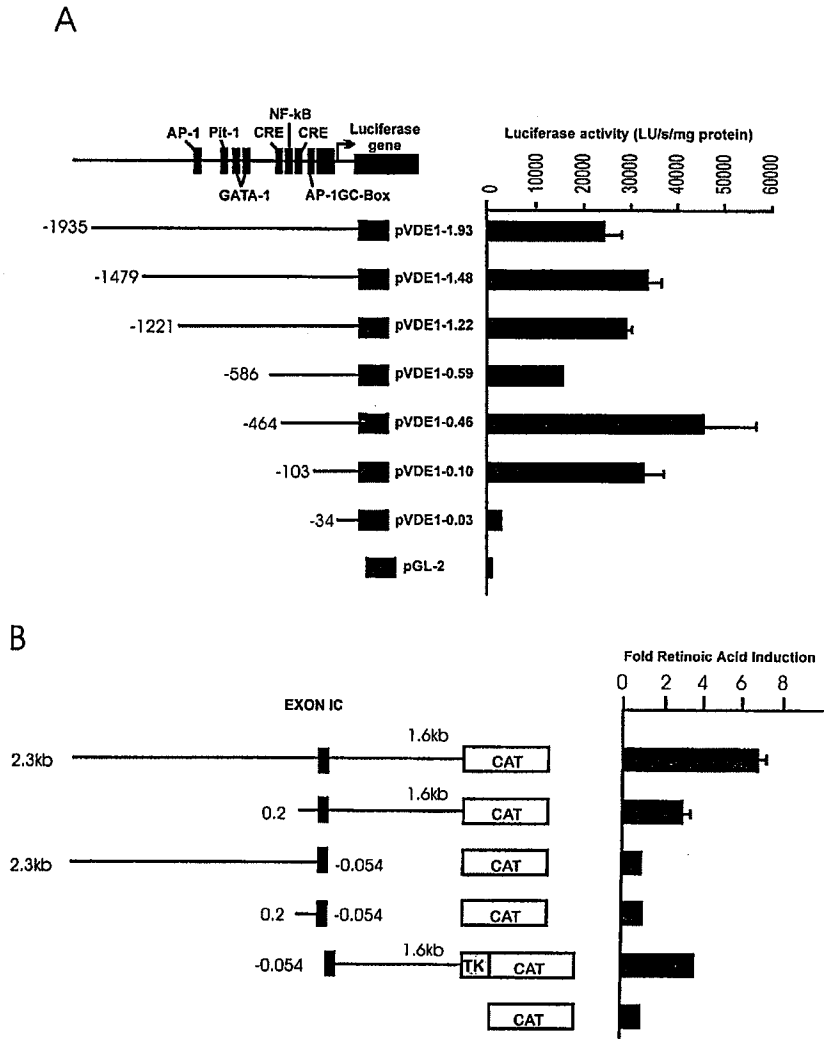

Fig. 5. Functional Analysis of the Human VDR Gene Promoter

A, hVDR promoter constructs pVDE1–1.93, pVDE1–1.48, pVDE1–1.22, pVDE1–0.589, pVDE1–0.46, pVDE1–0.10, pVDE1–0.03, and the promoterless control plasmid (pGL-2) were individually cotransfected together with the normalization vector pCMV-β-galactosidase as indicated in *Materials and Methods*. The activities of these constructs in Hela cells were determined after 64 h and normalized to the activity of β-galactosidase. Luciferase activity is expressed as light units per second per mg of protein. Data are means ± SEM of four independent experiments. B, DNA fragments surrounding exon 2 as indicated in the figure were fused to the pBL CAT2 or pBLCAT3 chloramphenicol acetyltransferase expression vectors and transfected into rat ROS 17/2.8 cells as described in *Materials and Methods*. Transfected cells were treated with either vehicle or retinoic acid (10^{-6} M) for 48 h after which the cells were harvested and extracts (100 μg protein) assayed for CAT activity. Each assessment represents the mean ± SEM of a triplicate determination. The data are representative of three separate experiments and are expressed as the activity of the construct in the presence of retinoic acid compared with the activity of the construct in the ligand's absence (fold induction).

A Retinoid-Responsive Region Lies Downstream of Exon 2

In view of the complexity of the hVDR gene, we examined several DNA fragments surrounding exon 1C for their capacity to direct transcriptional activity or to be regulated by hormones such as 1,25-$(OH)_2D_3$ or retinoic acid. All clones examined exhibited transcriptional activity when cloned into the promoterless chloramphenicol acetyltransferase (CAT) vector pBLCAT3 (data not shown). As observed in Fig. 5B, however, several constructs, each containing intron sequence downstream of exon 2, were responsive to treatment with retinoic acid (10^{-6} M). The constructs were uniformly unresponsive to 1,25-$(OH)_2D_3$ in this cell line (data not shown). The ability of this fragment of DNA to transfer retinoic acid response to the viral thymidine kinase promoter provides further support for the possibility that the previously identified regulation of VDR expression by retinoic acid (33, 34) is direct and mediated via a *cis*-element located a sig-

nificant distance downstream of the authentic promoter. Further work will be required to define the exact location of this *cis*-element.

DISCUSSION

We report here the structural organization of the human VDR gene derived from several λ-clones and four overlapping cosmid clones that span the entire gene locus. The human gene comprises 11 exons that together with the associated introns cover approximately 75 kb of DNA. Three exons (exons 1A, 1B, and 1C) make up the 5′-noncoding leader sequence of the largest of the hVDR mRNA species (type 2). An additional eight exons (exons 2–9) encode the structural portion of the gene product. This numbering system allows retention of the numbering system assigned in an earlier preliminary analysis of the gene (43) as well as that found in a body of literature related to the existence of hVDR gene polymorphisms that has evolved recently (see below). The multiple exonic structure and general organization of the hVDR gene are comparable to that of many of the other steroid receptor genes that have been characterized including the thyroid receptor (18), progesterone receptor (11), estrogen receptor (12), androgen receptor (14), and glucocorticoid receptor (13, 17). The promoter for this gene is TATA-less and GC rich.

The organization of the hVDR gene indicates that separate exons encode each of the zinc finger modules and that the 3′-boundaries of each of these exons appear to be generally conserved within the nuclear receptor family of genes. One exception may be chicken ovalbumin upstream promoter-transcription factor α and β, for which both finger modules appear to be encoded by a single exon (16). Although the two zinc modules within these proteins appear highly related structurally, they are not equivalent topologically (50). Furthermore, the function of each module is substantially different; the first zinc module determines the specificity of DNA binding whereas the second is more intimately involved in the protein-protein contacts that stabilize the association through dimerization (51). Although it is possible that the two exons encoding these modules evolved from a common ancestral gene through duplication and subsequently diverged under a different set of selective pressures, it is also possible that they evolved independently. Three exons encode the hVDR hinge, whereas only two encode this flexible region within the sex steroid receptor genes. It is noteworthy that the VDR appears to contain an extended hinge region relative to many of the other members of this gene family (4). The function, if any, of these additional hVDR residues that are encoded by insertion of exon 5 is unknown, although this region appears to be the least conserved among VDRs from different species. Finally, exon 2 of the hVDR is restricted to 21 amino acids upstream of the first zinc module, placing the hVDR DNA-binding domain near the amino-terminal end of the protein. This is unlike several of the other family members where one or more relatively large exons lie upstream, relegating the DNA-binding domain to a more central location within the protein (4). The absence of this extended region within the hVDR, whose presence in the larger receptors is associated with an important activation domain(s) (52–54), implies a more complete reliance of the hVDR on the carboxy-terminal activation function (AF2) or on an, as yet, undescribed activation region.

Amplification of the 5′-end of the hVDR mRNA revealed the existence of three separate transcripts in human kidney RNA. Because each contains the identical start site and first exons, the transcripts are likely derived from alternative splicing of two of the 5′-noncoding exons, 1B and 1C, one of which eliminates exon 1B and the other which eliminates both exons 1B and 1C. It is hypothetically possible that a fourth transcript that does not contain exon 1C also exists, perhaps in tissues other than kidney. Alternative splicing of the RAR, retinoid X receptor, thyroid hormone receptor, and other receptors is a common feature of this gene family of proteins. Unlike that which was observed here, some splicing events lead to the productions of different proteins with unique functions (19, 20). The relative abundance of each of the three hVDR transcripts within the kidney is unknown; indeed, the similarity in their size prevented their earlier detection by Northern blot analysis. The existence and relative abundance of these hVDR transcripts in other tissues as well as a determination of their possible individual functions remain for future studies.

The promoter region of the hVDR lies in a GC-rich island and does not contain a TATA box. In that respect, the hVDR gene is like certain other steroid receptor gene promoters. Our analysis of this promoter indicates a substantial capacity to direct transcription of a chimeric reporter function. The most proximal region (−103 to −34) imparts more than 80% of the activity of the promoter in HeLa cells and appears to contain strong positive elements. A possible negative contribution is evident between −586 and −464. The hVDR promoter contains an array of putative binding sites for transcription factors that mediate the activities of multiple pathways that serve to transduce a variety of extracellular signals. For example, it is known that the VDR is regulated by both PKA and PKC pathways (27–29, 55, 56) that are in turn known to converge on several specific transcription factors. These and other studies support the possibility that binding sites upstream of the hVDR promoter may play modulatory roles in its regulation, although future functional studies will be necessary to confirm this hypothesis. At the very least, the presence of five GC boxes immediately upstream of the start site of transcription suggests a fundamental role for the transcription factor SP1 in the activation of this gene (47). SP1 is known to interact both with a number of cellular and viral promoters as well as with other transcription

factors such as NFkB (57, 58). Interestingly, the hVDR gene is induced with retinoic acid at a site that lies downstream of exon 1C. While a potential binding site for one or more of the retinoid receptors has not been definitively localized, it is likely that this site mediates the recognized ability of RA to induce the transcription of VDR (33, 34). The existence and, more importantly, the location of this site, together with the size of the introns lying between exons 1A, 1B, and 1C, suggest that it may prove difficult technically to identify additional regulatory sequences. The inability to detect vitamin D inducibility of the VDR gene at the promoter level currently suggests that the autoregulatory actions of vitamin D are indirect. For example, a recent study suggests that 1,25-$(OH)_2D_3$ can stimulate the expression of c-*fos* in osteoblastic cells that might in turn stimulate hVDR gene expression (59). Based upon the noted complexity at the 5′-end of the VDR gene, however, it is possible that 1,25-$(OH)_2D_3$-responsive portions of the gene have yet to be defined.

During the analysis of the hVDR gene, we identified the existence of a polymorphism associated with exon 2. This polymorphism leads to synthesis of two different hVDR mRNAs which we detected utilizing single-strand conformational analysis (46). One corresponds to the mRNA sequence of Baker *et al.* (42) wherein the start site of translation is situated at codon 1 and the second is associated with a T to C conversion in this codon that results in potential translation beginning at an in-frame ATG located downstream at codon 4. Translation of such an mRNA would result in the production of an abbreviated protein of only 424 amino acids. Two studies have recently reported the distribution of these alleles in the human population (60, 61); the ATG to ACG allele, which can be detected by the presence of a *Fok*I site (60), represents the more common form. Interestingly, the frequency of this allele correlates with an increase in bone density in two different human female populations (60, 61). This observation suggests that the VDR products of these two alleles may exhibit unique activities. Validation of this hypothesis, however, will require additional population studies as well as the demonstration that the two VDR alleles are indeed expressed in human tissues and that they exhibit quantitative and/or qualitative differences in their activity on bone or other tissues. As a first step, Arai *et al.* (61) have shown that both proteins can be produced through recombinant means in transfected COS cells. More importantly, the capacity of these two gene products to direct transcription of a vitamin D-sensitive chimeric gene in cotransfected cells is quantitatively different. The smaller protein, whose corresponding allele appears to correlate with increased bone density, exhibited greater transcriptional activity. Additional studies will be necessary to confirm these functional studies as well as the correlation between bone mineral density and VDR alleles. Interestingly, some plasticity is also apparent in these two codons in other species; while the mouse VDR mRNA contains both ATGs (62), the rat VDR mRNA contains only the most 5′-ATG (63). Perhaps unique activities are also associated with these mRNA products.

Additional polymorphisms have been identified within the hVDR gene in the intron between exons 7 and 8 (*Bsm*I and *Apa*I) and within the 3′-noncoding sequences lying in exon 9 (*Taq*I) (64) (see Table 3). The presence of these restriction fragment length polymorphisms has been reported to be associated with population bone mineral density (65) and more recently with prostate cancer (66). In the former case, this finding has not been widely reproduced, suggestive of relatively weak linkage, and remains highly controversial (67). In contrast to the *Fok*I polymorphism located in exon 2, it is unclear how the *Bsm*I and/or *Taq*I polymorphisms located in noncoding portions of the hVDR gene might influence hVDR function. It is possible that they are linked in some way either to the potentially functional exon 2 start site polymorphism, to as yet unidentified allelic differences located within the hVDR gene promoter, or to unrelated genes that impact bone mineralization directly. Both the validity of the proposed associations between hVDR polymorphisms and disease and, if proven true, the mechanism(s) by which they impart disease remain to be established.

In conclusion, we report on the structural organization of the hVDR chromosomal gene. The availability of this gene locus as well as identification and cloning of its promoter will enable future studies aimed at identifying molecular determinants of the VDR's expression. A wealth of studies that describe the regulation of expression of the VDR provide the backdrop and rationale for these impending studies.

MATERIALS AND METHODS

Messenger RNA Analysis

The sequence of the 5′-end of the human VDR mRNA was determined utilizing the 5′-RACE system obtained from Life Technologies, Inc, (Gaithersburg, MD) (68). Total RNA was isolated from human kidney (69, 70) and used to synthesize first-strand cDNA utilizing random hexamers and oligo d(T) primers and Superscript RT (Life Technologies, Inc). Two sequential PCR amplification reactions were employed to identify the 5′-ends of the hVDR mRNAs and to generate DNA fragments useful for initial sequencing. As illustrated in Fig. 1A, the first was performed using common anchored primer A (5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3′) and primer B2 (5′-CTTCCGCTTCAGGATCATCTCCCGC-3′), which corresponds to nt +309 to +333 relative to the translation start site of the human VDR cDNA of Baker *et al.* (42). The second was performed with a portion of the reaction products from the first amplification and used primer A and primer B1 (5′-GCAGGGGGCAGGTAAGTGG-3′), which corresponds to nt −29 to −11 relative to the start site of translation reported by Baker *et al.* (42). Sequence determination of the PCR products of these sequential reactions established the 5′-end of the hVDR mRNA transcript, enabling a subsequent amplification of kidney RNA using primer A1, which corresponds to the first 22 bases of the

authentic 5′-end of the transcript and primer B2 (see Fig. 1A). All amplified DNA products were subcloned into pBluescript II SK (Strategene, San Diego, CA) and sequenced using the SequiTherm Long-Read Cycle Sequencing Kit-LC (EpiCentre Technologies, Madison, WI). All DNA sequencing was performed using the fluorescence-based LI-COR model 4000L sequencer.

Recovery and Analysis of hVDR Genomic Clones

A human EMBL 3 genomic library was screened with ^{32}P-labeled PCR probes obtained after 5′-RACE of human kidney mRNA using high-stringency hybridization conditions. Nylon membrane replicas of approximately 1.5×10^6 plaques were incubated for 2 to 3 h in 50% formamide, 5× Denhardt's solution, 0.75 M NaCl, 0.05 M NaH_2PO_4, pH 7.4, 3 mM EDTA, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Hybridization was performed overnight at 42 C with 5×10^5 cpm/ml labeled probe. Membranes were washed for 1 h at 30 C in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0, and 0.05% SDS and then twice for 1 h at 68 C in 0.15 M sodium citrate, pH 7.0, and 0.1% SDS. After exposure of the membranes to Kodak X-Omat film, positive plaques were isolated, rescreened twice, and purified, and then the inserts were isolated and subcloned into pBlueScript II SK. DNA inserts were verified by Southern blot analysis, restriction enzyme-mapped by routine methods, and portions were then sequenced using the strategy outlined below.

A human liver genomic library prepared in the cosmid vector pCV109 (44) was screened via colony hybridization techniques (71) using nick-translated hVDR cDNA probes. HVDR probe 1 extended from −115 to +145 nt relative to the sequence of Baker *et al.* (42); hVDR probe 2 contained nucleotides +10 through +576 relative to the sequence of Baker *et al.* (42); and hVDR probe 3 contained nucleotides +10 through +2012 nt relative to the sequence of Baker *et al.* (42). The latter probe represents the entire open reading frame of the normal hVDR cDNA together with 731 nucleotides of noncoding 3′-sequence. Nylon filter-immobilized DNA was prehybridized at 68 C overnight in 6× NaCl-sodium citrate (SSC) (1× = 0.15 M $NaCl_2$, 15 mM sodium citrate, pH 7), 2 mM EDTA/0.5% nonfat dry milk (wt/vol), and then hybridized with ^{32}P-labeled probes (initially probes 2 and 3) under identical conditions for 16 h. Filters were washed for 2 h with several changes of 0.15 M $NaCl_2$, 15 mM sodium citrate, pH 7, 0.5% SDS at 68 C and then autoradiographed overnight. Positive phVDRG cosmid clones were isolated through three to four rounds of additional screening.

Human genomic DNA was prepared as described (71). The latter as well as cosmid phVDRG DNA were transferred to nylon membranes, prehybridized for 6 h at 68 C in 6× SSC, and then hybridized with the appropriate ^{32}P-labeled hVDR cDNA probes overnight. Filters were washed for 2 h as above, and autoradiographed for 4 h (cloned DNA) or 48 h (human genomic DNA). phVDRG DNA was isolated via alkaline lysis and polyethylene glycol precipitation techniques as previously described (71). The isolated DNA was mapped with restriction endonucleases by routine techniques. The relative orientation of specific DNA fragments of related size within the clones was determined by sequential probing with hVDR cDNA probes 1, 2, and 3. DNA fragments that hybridized with the hVDR cDNA probes were isolated, subcloned into the pGEM 3 cloning vector (Promega Biotech, Madison, WI), and then subjected to standard sequencing methods using Sequenase (US Biochemical Corp., Cleveland, OH). The sequencing strategy we employed involved determining the ends of each DNA fragment utilizing the T7 and SP6 sequencing primers followed by extension of this sequence with synthetic oligonuclotides complementary to the newly identified sequence. As the relative position of hVDR gene exons emerged within the DNA clones, synthetic oligonucleotides corresponding to hVDR cDNA sequence either 5′ or 3′ to the identified exon were used as sequencing primers. Intron/exon boundaries were identified using pairs of primers that generated sequence from both strands. Orientation of the exon(s) within the DNA fragment was achieved by identifying sequence overlaps within the clone or by mapping a unique restriction site identified within the newly obtained sequence.

hVDR Gene Promoter Analysis

hVDR promoter constructs were prepared beginning with an approximately 3.2 kb *SacI-Hin*dIII fragment of the hVDR gene extending from exon 1 upstream approximately −3200 bp. This fragment (pVDE1–3.2) as well as additional fragments were cloned into the *Hin*dIII site of the luciferase expression vector pGL-2 basic (Promega). Plasmids pVDE1–2.6, pVDE1–1.93, pVDE1–1.48, pVDE1–1.22, pVDE1–0.59, pVDE1–0.46, pVDE1–0.10, and pVDE1–0.03 were similarly constructed from promoter fragments that contained common 3′-ends but extended 2.6, 1.93, 1.22, 0.586, 0.462, 0.10, or 0.03 kb upstream, respectively. DNA restriction fragments were also isolated from hVDR gene DNA surrounding exon 1C. These fragments include exon 1C and the length of intron sequence lying both upstream and/or downstream of exon 1C as indicated in the figure. The activity of these fragments of DNA was assessed in promoterless pBL-CAT3 and thymidine kinase promoter-containing pBL-CAT2 chloramphenicol acetyltransferase expression vectors (72). The orientation and cloning boundaries of all constructs were verified through DNA sequence analysis.

Transient Transfection Analysis of hVDR Promoter Sequences

Hela cells were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and nonessential amino acids. Rat osteosarcoma ROS 17/2.8 cells were grown in Ham's F12 medium supplemented with 10% FCS containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), and nonessential amino acids as previously described. Cells were transfected with DNA 24 h after passage using either polybrene (73) (Sigma Chemical Co., St. Louis, MO) (ROS 17/2.8) or lipofectAMINE (Life Technologies Inc.) (HeLa cells). hVDR gene DNA reporter plasmids (5 or 10 μg) were cotransfected together with 1 μg β-galactosidase normalization vector (1 μg), and the activities of the enzymes CAT, luciferase, and/or β-galactosidase were evaluated in cellular extracts prepared 64 h following transfection. Luciferase or CAT activities were determined as previously described (65, 74) and normalized to the activity of β-galactosidase (75). All plasmids used for cellular transfections were purified on Qiagen ion exchange columns.

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